TRANSLATION

WESTPHALIA UNIVERSITY
INSTITUTE OF PHARMACOLOGY AND TOXICOLOGY
DOMAGKSTR. 12 · 4400 MÜNSTER

REPORT
ON
TOXICITY TESTS
ON
INCUBATED CHICKEN EGGS

(HET - EMBRYO TOXICITY - TEST)

TEST SUBSTANCE : LUSANTAN T 3
BEIERSDORF AG

F.H. KEMPER

N.P. LEUPKE

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[signed]
Prof. Dr. F.H. Kemper
Director of the Institute

[signed]
Prof. Dr. N.P. Luepke
Head of testing

1. S U M M A R Y

1. S U M M A R Y

The test substance Lusantan T3 was injected, dissolved or suspended in olive oil, in a volume of 0.1 ml/egg on d1 or d5 into battery eggs (White Leghorn; Lohmann Selected Leghorn, LSL).

The spontaneous hatching rates of the surviving embryos were high, but there were found to be some slight changes in the following:

I embryolethality
II retardation
III systemic toxicity

At the highest doses (d1: 25.0 mg/egg; d5: 10.0 mg/egg), less than 50 % of the embryos died; i.e. the acute embryotoxicity as median lethal dose (LD_{50}) is:

d1 above 25.0 mg/egg; above 500 ppm
d5 above 10.0 mg/egg; above 200 ppm.

A computed extrapolation yields the following:

d1 about 45.0 mg/egg; about 900 ppm
d5 about 25.0 mg/egg; about 500 ppm.

The acute toxicity is therefore classified as very moderate.

The effects of retardation and systemic toxicity found in a few cases with the higher doses were associated with an increase in embryolethality, but particular findings which could be related to the test substance were not observed.

There was no increased occurrence, compared with historical findings or control groups, in gross abnormalities or malformations in embryos which died or hatched chicks after administration of the test substance Lusantan T3 either on d1 or on d5.

Taking account of the spontaneous death rate, normal ranges and comparison with control groups, the HET embryotoxicity test revealed the following no-effect level for the test substance Lusantan T3:

d1 2.5 mg/egg
d5 1.0 mg/egg.

2. TEST CONDITIONS

2. TEST CONDITIONS

2.1 Test substance

The test substance was provided under the name Lusantan T3 by Beiersdorf AG, D-2000 Hamburg 20, for toxicity tests on incubated chicken eggs (hen's egg test, HET) in an amount of about 50 g in an uncolored wide-necked glass bottle with screw cap and the above labelling.

The test substance was a white, caking powder of melting point 100 - 101°C; batch No. 17745/104. No other physical, chemical or physicochemical data were provided.

According to Beiersdorf AG, Lusantan T3 is a "solid solution" of 2,4,6-tris(p-2-ethylhexoxycarbonylanilino)-s-triazine (Mol. wt. 823) and 2-ethylhexanoic acid C16/C18 ester in the ratio 63.5 % triazine to 36.5 % ester, which is intended to be used as sunscreen agent (UV filter) in cosmetic compositions.

2.2. Livestock material

All the investigations were carried out on fresh hatchery eggs laid by hybrids of the white leghorn breed of the same, genetically controlled breeding strain (Lohmann Selected Leghorn, LSL), which were obtained on the date of laying from a commercial breeding unit (J. Brinkschulte, Gut Aversfeld, Senden/Westf.). Only eggs weighing 50 - 60 g were used. The eggs were stored with the pointed end downwards in a cooled room for 24 hours so that the air bubble was positioned under the round end of the egg, with the yolk and the sensitive embryo underneath. Before the start of the test, the eggs were candled, and eggs with defective shells were eliminated.

2.3. Administration method and incubation

The test substance was dissolved or suspended in an appropriate concentration in olive oil (DAB) under aseptic conditions on a clean bench.

After the eggs had been stored with the round end at the top under cool conditions without being moved for 24 hours, the pointed end of the shell was disinfected with an alcohol swab and, with the egg horizontal, an electric crown drill was used to drill a hole with a diameter of about 1 mm at the pointed end, slightly offset, taking care not to damage the embryonic membrane. The preparation of test substance was instilled using a tuberculin syringe (0.05 ml graduations) through a metal needle (No. 14) slowly and cautiously into the egg white through this hole at the pointed end. The volume injected was always 0.1 ml/egg; control groups received only the olive oil vehicle. The hole bored in the eggshell was closed after administration with filler (Dufix^{R}).

The test substance was administered in two test series on d1, i.e. before the start of incubation, and on d5, i.e. 96 hours after the start of incubation.

The eggs were incubated in a Schumacher incubation unit with continuous circulation of air initially at 37.5°C (± 1.0°C) and a relative humidity of 70.0 % (± 10.0 %) for 17 days; this corresponds to optimized incubation conditions. The eggs were turned automatically every two hours. From day 5 of incubation onward, the eggs were candled with a mercury vapor lamp at intervals of one to two days until transferred into the hatcher on day 18.

Disturbed development or death of the embryos can be detected early by candling in particular from the condition of the blood vessels and the movements of the embryo in the amnion. All eggs found to be abnormal or with dead embryos on candling were opened, and the stage of development was determined by inspection using comparison tables (HAMBURGER and HAMILTON). Particular attention was paid to gross malformations.

In the hatcher, the eggs were kept at 38.5°C (± 0.8°C) and a relative humidity of 80.0 % (± 10.0 %) from day 18 of incubation until hatching. Table 1 shows the incubation conditions.

2.4. Test parameters

Most of the chicks hatched in the night from day 21 to day 22 of incubation, but small numbers hatched during day 21 or day 22. At the end of day 22 of incubation, the eggs which had not hatched were opened and the embryos were inspected. The stage of development was determined using the comparison tables mentioned above, and any malformations were noted. The birds were removed from the hatcher and their viability and maturity were determined (regular breathing, yolk sac retraction, ability to stand and run). The birds were examined for gross malformations (especially of the beak, eyes, skull, wings, legs and feet); clarification and staining were carried out for malformations which could not be classified on close inspection.

After determination of the hatching weight, the birds were sacrificed by decapitation under ether anesthesia. The largest frontal diameter of the skull was determined using a slide gage (9.1 mm graduation). Exsanguination was followed by preparation of the right upper and lower limbs and determination of the following bone lengths (0.5 mm graduation): humerus, ulna, femur, tibia, metatarsus.

After opening up, all the body cavities were inspected in situ. The liver and heart were removed to determine their weights using micro and torsion balances.

Serum was prepared from the blood of the test birds, and the following parameters were determined on this using a Technikon autoanalyzer:

> Sodium, potassium, calcium, chloride, inorganic phosphate, total protein, creatinine, glucose, uric acid, cholesterol, triglycerides, aspartate aminotransferase (GOT) alanine aminotranferase (GPT), lactate dehydrogenase (LDH), alkaline phosphatase (AP).

Statistical analysis was carried out with a Hewlett-Packard computer. Significances were calculated with a Mann-Whitney-Wilcoxon test.

3. TEST RESULTS

3. TEST RESULTS

The investigations and the results are tabulated and shown in graphs in an annex (4.); references to tables below as a rule refer to this annex.

3.1. Embryolethality

The test substance Lusantan T3 was injected, dissolved or suspended in olive oil in a volume of 0.1 ml/egg on d1 or d5 into hatchery eggs (see Tab. 2). The hatching rates of the surviving embryos (see Tab. 3) were high but there were slightly dose-dependent mortality rates in both test series (see Tab. 3, 4); with the highest doses (d1: 25.0 mg/egg; d5: 10.0 mg/egg), less than 50 % of the embryos died; i.e. the acute embryotoxicity as median lethal dose (LD_{50}) is:

d1 above 25.0 mg/egg; above 500 ppm
d5 above 10.0 mg/egg; above 200 ppm.

A LITCHFIELD and WILCOXON extrapolation yielded the following:

d1 about 45.0 mg/egg; about 900 ppm
d5 about 25.0 mg/egg; about 500 ppm.

Thus the test substance Lusantan T3 is classified as having a very moderate acute toxicity.

3.2. Retardation

The test substance Lusantan T3 was injected, dissolved or suspended in olive oil, in a volume of 0.1 ml/egg on d1 or d5 into hatchery eggs (see Tab. 2). The hatching rates of the surviving embryos (see Tab. 3) were high but there were a few signs of retardation in the test parameters of hatching weight, skull thickness and bone lengths (humerus, ulna, femur, tibia, metatarsus) as the dose increased (see Tab. 4), as a rule associated with an increase in embryo lethality (see Tab. 3). These findings must be viewed in the context both of the control groups and of the normal ranges obtained from a large number of control findings (see Tab. 5).

There were no special abnormalities.

3.3. Systemic toxicity

The test substance Lusantan T3 was injected, dissolved or suspended in olive oil, in a volume of 0.1 ml/egg on d1 or d5 into hatchery eggs (see Tab. 2). The hatching rates of the surviving embryos (see Tab. 3) were high, but there were a few changes in the test parameters.

These findings must be viewed in the context both of the control groups and of the normal ranges obtained from a large number of control findings (see Tab. 5).

These changes are assessed as unrelated to the dose and within the normal ranges.

3.4. Teratogenicity

There was no increase in the occurrence of gross abnormalities or malformations, compared with historical findings and control groups, in embryos which died or hatched chicks after administration of the test substance Lusantan T3 either on d1 or on d5.

4. A N N E X

HET CODE : BG

SUBSTANCE CODE : Lusantan T3

Inj. : d1

d	Humidity (%)	Temp. (°C)	Hatch hum/temp
1	78	37.8	
2	81	37.9	
3			
4	70	38.2	
5			
6			
7	68	38.0	
8	78	38.0	
9	75	37.5	
10	70	37.6	
11	68	38.3	
12			
13			
14	60	37.4	
15	74	38.5	
16	75	38.0	
17	60	37.6	
18	70	37.9	90/38.9
19			
20			85/38.5
21			85/39.0

Table 1

HET CODE : BE

SUBSTANCE CODE : Lusantan T3

Inj. : d5

d	Humidity (%)	Temp. (°C)	Hatch hum/temp
1	74	36.9	
2	75	37.8	
3	64	37.3	
4	66	37.8	
5	79	37.4	
6			
7			
8	72	37.5	
9	79	38.0	
10	76	38.1	
11	81	37.6	
12	78	37.5	
13			
14			
15	71	38.3	
16	78	37.8	
17	81	37.9	
18	75	37.7	
19	70	38.0	85/38.4
20			80/38.7
21			90/38.6

Table 1

Table 2 : List of tests carried out
Volume administered 0.1 ml in each case
Solution or suspension in olive oil

Substance	d	Dose/egg (mg)	n
LT 3	1	0.0	22
		1.0	20
		2.5	20
		10.0	20
		25.0	15
	5	0.00	25
		0.25	20
		1.00	20
		2.50	20
		10.00	20

d Day of administration
n Number of eggs used

Table 3 : Summary of the mortality in ovo
Test substance

d	Group	n	n.f.	I	1-7	8-12	13-17	18-21
1	C	22	4	-	-	-	-	-
	I	20	-	-	1	-	-	2
	II	20	-	-	2	-	-	2
	III	20	1	-	1	1	-	5
	IV	15	1	-	-	1	-	4
5	C	25	4	-	-	1	2	2
	I	20	4	-	2	2	-	1
	II	20	1	-	3	1	1	1
	III	20	3	-	1	2	2	1
	IV	20	-	-	2	2	-	4

C 0.1 ml olive oil
I 1.0 (0.25) Lusantan T 3/egg
II 2.5 (1.00) Lusantan T 3/egg
III 10.0 (2.50) Lusantan T 3/egg
IV 25.0 (10.00) Lusantan T 3/egg

n.f. = not fertilized
I = infection

HET CODE : BG

SUBSTANCE CODE : Lusantan T3

Inj. d 1

LD_{50} mg/egg

Parameter		0.0 mg/egg	1.0 mg/egg	2.5 mg/egg	10.0 mg/egg
Mortality (%)			15	20	36.8
Survival rate (%)		100	85	80	63.2
Bodyweight (g)	$\bar{x}$	42.33	41.31	42.99	45.03
	sd	2.84	3.43	2.96	2.84
	se	0.67	0.83	0.74	0.86
Liver weight	$\bar{x}$	0.86	0.88	0.89	0.86
absolute (g)	sd	0.08	0.10	0.07	0.09
	se	0.02	0.02	0.02	0.03
Liver weight	$\bar{x}$	2.04	2.14	2.06	1.92
relative	sd	0.22	0.27	0.13	0.21
g/100 g bodyweight	se	0.05	0.07	0.03	0.06
Heart weight	$\bar{x}$	0.28	0.25	0.25	0.26
absolute (g)	sd	0.03	0.04	0.03	0.02
	se	0.01	0.01	0.01	0.01
Heart weight	$\bar{x}$	0.65	0.61	0.58	0.57
relative	sd	0.07	0.11	0.06	0.05
g/100 g bodyweight	se	0.02	0.03	0.01	0.02

x p 0.01

xx p 0.005

xxx p 0.001

Table 4

HET CODE : BG

SUBSTANCE CODE : Lusantan T3

Inj. d 1

LD_{50} mg/egg

Parameter		25.0 mg/egg	mg/egg	mg/egg	mg/egg
Mortality (%)		35.7			
Survival rate (%)		64.3			
Bodyweight (g)	$\bar{x}$	42.58			
	sd	2.00			
	se	0.67			
Liver weight	$\bar{x}$	0.84			
absolute (g)	sd	0.09			
	se	0.03			
Liver weight	$\bar{x}$	1.98			
relative	sd	0.23			
g/100 g bodyweight	se	0.08			
Heart weight	$\bar{x}$	0.25			
absolute (g)	sd	0.03			
	se	0.01			
Heart weight	$\bar{x}$	0.60			
relative	sd	0.08			
g/100 g bodyweight	se	0.03			

x p 0.01
xx p 0.005
xxx p 0.001

HET CODE : BG SUBSTANCE CODE : Lusantan T3

Inj. d 1

Parameter		0.0 mg/egg	1.0 mg/egg	2.5 mg/egg	10.0 mg/egg
Skull diameter (mm)	$\bar{x}$	13.2	13.2	13.4	13.3
	sd	0.2	0.3	0.2	0.2
	se	0.1	0.1	0.1	0.1
Humerus (mm)	$\bar{x}$	17.7	17.7	17.7	17.7
	sd	0.7	0.5	0.5	0.5
	se	0.2	0.1	0.1	0.1
Ulna (mm)	$\bar{x}$	18.1	17.9	18.1	18.2
	sd	0.5	0.7	0.6	0.8
	se	0.1	0.2	0.1	0.2
Femur (mm)	$\bar{x}$	23.4	23.2	23.4	23.6
	sd	0.5	0.7	0.8	0.7
	se	0.1	0.2	0.2	0.2
Tibia (mm)	$\bar{x}$	33.4	33.0	33.6	33.3
	sd	0.9	0.9	0.6	0.7
	se	0.2	0.2	0.2	0.2
Metatarsus (mm)	$\bar{x}$	24.9	24.7	25.1	25.6
	sd	1.4	1.4	1.3	1.3
	se	0.3	0.3	0.3	0.4

x p 0.01
xx p 0.005
xxx p 0.001

HET CODE : BG

SUBSTANCE CODE : Lusantan T3

Inj. d 1

Parameter		25.0 mg/egg	mg/egg	mg/egg	mg/egg
Skull diameter (mm)	$\bar{x}$	13.3			
	sd	0.2			
	se	0.1			
Humerus (mm)	$\bar{x}$	17.4			
	sd	0.5			
	se	0.2			
Ulna (mm)	$\bar{x}$	18.2			
	sd	0.4			
	se	0.2			
Femur (mm)	$\bar{x}$	23.1			
	sd	0.6			
	se	0.2			
Tibia (mm)	$\bar{x}$	33.2			
	sd	0.8			
	se	0.3			
Metatarsus (mm)	$\bar{x}$	25.0			
	sd	1.2			
	se	0.4			

x p 0.01
xx p 0.005
xxx p 0.001

HET CODE : BG SUBSTANCE CODE : Lusantan T3

Inj. d 1

Biochemical parameter		0.0 mg/egg	1.0 mg/egg	2.5 mg/egg	10.0 mg/egg
Tot. Bilirubin (mg/dl)	$\bar{x}$				
	sd				
	se				
GOT (U/l (25°))	$\bar{x}$	83.40	93.25	85.40	84.7
	sd	8.56	2.63	4.10	2.8
	se	3.83	1.31	1.83	1.4
GPT (U/l (25°))	$\bar{x}$	8.00	15.20	10.60	6.0
	sd	2.65	7.82	4.28	6.2
	se	1.53	3.50	1.91	3.1
LDH (U/l (25°))	$\bar{x}$	955.00	957.80	873.20	775.7
	sd	109.92	108.14	141.71	23.5
	se	49.16	48.36	63.38	11.7
AP (U/l (25°))	$\bar{x}$	1165.40	1432.00	1554.00	1440.0
	sd	608.57	189.00	179.53	193.3
	se	272.16	84.52	80.29	96.7
γ-GT (U/l (25°))	$\bar{x}$	3.80	3.80	3.80	4.0
	sd	0.45	0.45	0.84	0.0
	se	0.20	0.20	0.37	0.0

x p 0.01
xx p 0.005
xxx p 0.001

HET CODE : BG

SUBSTANCE CODE : Lusantan T3

Inj. d 1

Biochemical parameter		25.0 mg/egg	mg/egg	mg/egg	mg/egg
Tot. Bilirubin (mg/dl)	$\bar{x}$ sd se				
GOT (U/l (25°))	$\bar{x}$ sd se	83.00 7.16 3.58			
GPT (U/l (25°))	$\bar{x}$ sd se	4.75 2.50 1.25			
LDH (U/l (25°))	$\bar{x}$ sd se	805.25 102.85 51.43			
AP (U/l (25°))	$\bar{x}$ sd se	1672.50 82.61 41.31			
γ-GT (U/l (25°))	$\bar{x}$ sd se	4.25 0.50 0.25			

x p 0.01
xx p 0.005
xxx p 0.001

HET CODE : BG

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Inj. d 1

Biochemical parameter		0.0 mg/egg	1.0 mg/egg	2.5 mg/egg	10.0 mg/egg
Tot. protein (g/dl)	$\bar{x}$	1.80	1.94	1.72	1.60
	sd	0.20	0.21	0.08	0.24
	se	0.09	0.09	0.04	0.12
Creatinine (mg/dl)	$\bar{x}$	0.32	0.30	0.32	0.30
	sd	0.04	0.00	0.04	0.00
	se	0.02	0.00	0.02	0.00
Glucose (mg/dl)	$\bar{x}$	258.20	242.60	261.80	250.25
	sd	18.31	5.50	16.48	2.63
	se	8.99	2.46	7.37	1.31
Uric acid (mg/dl)	$\bar{x}$	4.14	4.40	5.42	5.14
	sd	0.60	0.69	1.75	0.71
	se	0.27	0.31	0.78	0.36
Cholesterol (mg/dl)	$\bar{x}$	390.60	393.80	383.40	366.50
	sd	20.55	15.25	20.51	16.78
	se	9.19	6.82	9.17	8.39
Triglycerides (mg/dl)	$\bar{x}$	101.60	98.40	101.00	99.75
	sd	14.26	22.69	9.51	10.53
	se	6.38	10.15	4.25	5.27

x p 0.01
xx p 0.005
xxx p 0.001

HET CODE : BG

SUBSTANCE CODE : Lusantan T3

Inj. d 1

Biochemical parameter		25.0 mg/egg	mg/egg	mg/egg	mg/egg
Tot. protein (g/dl)	$\bar{x}$	1.75			
	sd	0.40			
	se	0.20			
Creatinine (mg/dl)	$\bar{x}$	0.38			
	sd	0.05			
	se	0.03			
Glucose (mg/dl)	$\bar{x}$	250.25			
	sd	17.08			
	se	8.45			
Uric acid (mg/dl)	$\bar{x}$	4.33			
	sd	0.43			
	se	0.22			
Cholesterol (mg/dl)	$\bar{x}$	396.25			
	sd	34.97			
	se	17.49			
Triglycerides (mg/dl)	$\bar{x}$	110.50			
	sd	19.33			
	se	9.67			

x p 0.01
xx p 0.005
xxx p 0.001

HET CODE : BG

SUBSTANCE CODE : Lusantan T3

Inj. d 1

Biochemical parameter		0.0 mg/egg	1.0 mg/egg	2.5 mg/egg	10.0 mg/egg
Sodium	$\bar{x}$	138.40	139.60	139.40	140.25
(mmol/l)	sd	2.30	1.52	1.52	0.96
	se	1.03	0.68	0.68	0.48
Potassium	$\bar{x}$	6.60	5.16	5.06	4.70
(mmol/l)	sd	1.19	0.77	1.01	0.53
	se	0.53	0.34	0.45	0.26
Calcium	$\bar{x}$	2.69	2.59 **	2.62	2.57
(mmol/l)	sd	0.04	0.03	0.09	0.08
	se	0.02	0.01	0.04	0.04
Chloride	$\bar{x}$	109.20	109.20	108.60	106.00
(mmol/l)	sd	2.59	4.66	4.51	4.08
	se	1.16	2.08	2.01	3.04
Inorg. phosphate	$\bar{x}$	5.72	5.36	5.06	5.05
(mg/dl)	sd	0.50	0.29	0.32	0.19
	se	0.22	0.13	0.14	0.10

x p 0.01
xx p 0.005
xxx p 0.001

HET CODE : BG

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Inj. d 1

Biochemical parameter		25.0 mg/egg	mg/egg	mg/egg	mg/egg
Sodium (mmol/l)	$\bar{x}$	140.25			
	sd	4.72			
	se	2.36			
Potassium (mmol/l)	$\bar{x}$	5.18			
	sd	1.37			
	se	0.69			
Calcium (mmol/l)	$\bar{x}$	2.70			
	sd	0.09			
	se	0.05			
Chloride (mmol/l)	$\bar{x}$	105.75			
	sd	5.56			
	se	2.78			
Inorg. phosphate (mg/dl)	$\bar{x}$	5.50			
	sd	0.98			
	se	0.49			

x p 0.01
xx p 0.005
xxx p 0.001

HET CODE : BE

SUBSTANCE CODE : Lusantan T3

Inj. d 5

LD_{50} mg/egg

Parameter		0.0 mg/egg	0.25 mg/egg	1.0 mg/egg	2.5 mg/egg
Mortality (%)		23.8	31.3	31.6	35.3
Survival rate (%)		76.2	68.7	68.4	64.7
Bodyweight (g)	$\bar{x}$	44.36	41.41 **	40.86	41.77
	sd	3.10	4.06	2.66	3.46
	se	0.89	1.35	0.74	1.09
Liver weight	$\bar{x}$	0.89	0.85	0.90	0.87
absolute (g)	sd	0.13	0.12	0.11	0.10
	se	0.04	0.04	0.03	0.03
Liver weight	$\bar{x}$	2.01	2.02	2.21	2.06
relative	sd	0.33	0.29	0.28	0.25
g/100 g bodyweight	se	0.09	0.10	0.08	0.08
Heart weight	$\bar{x}$	0.26	0.25	0.27	0.27
absolute (g)	sd	0.03	0.02	0.03	0.04
	se	0.01	0.01	0.01	0.01
Heart weight	$\bar{x}$	0.58	0.60	0.66	0.65
relative	sd	0.08	0.05	0.10	0.09
g/100 g bodyweight	se	0.02	0.02	0.03	0.03

x p 0.01
xx p 0.005
xxx p 0.001

HET CODE : BE

SUBSTANCE CODE : Lusantan T3

Inj. d 5

LD_{50} mg/egg

Parameter		10.0 mg/egg	mg/egg	mg/egg	mg/egg
Mortality (%)		40			
Survival rate (%)		60			
Bodyweight (g)	$\bar{x}$	42.37			
	sd	2.28			
	se	0.66			
Liver weight	$\bar{x}$	0.87			
absolute (g)	sd	0.11			
	se	0.03			
Liver weight	$\bar{x}$	2.10			
relative	sd	0.38			
g/100 g bodyweight	se	0.11			
Heart weight	$\bar{x}$	0.27			
absolute (g)	sd	0.03			
	se	0.01			
Heart weight	$\bar{x}$	0.65			
relative	sd	0.09			
g/100 g bodyweight	se	0.03			

x p 0.01
xx p 0.005
xxx p 0.001

HET CODE : BE

SUBSTANCE CODE : Lusantan T3

Inj. d 5

Parameter		0.0 mg/egg	0.25 mg/egg	1.0 mg/egg	2.5 mg/egg
Skull diameter (mm)	$\bar{x}$	13.5	13.4	13.4	13.5
	sd	0.3	0.5	0.4	0.3
	se	0.1	0.2	0.1	0.1
Humerus (mm)	$\bar{x}$	18.4	18.1	18.3	18.7
	sd	0.9	1.1	1.0	0.5
	se	0.3	0.4	0.3	0.2
Ulna (mm)	$\bar{x}$	17.6	17.8	17.6	18.0
	sd	0.8	1.0	1.0	0.7
	se	0.2	0.3	0.3	0.2
Femur (mm)	$\bar{x}$	23.8	23.6	23.7	23.7
	sd	0.9	1.1	1.0	1.2
	se	0.3	0.4	0.3	0.4
Tibia (mm)	$\bar{x}$	34.0	33.7	33.4	33.9
	sd	1.6	1.5	2.1	1.0
	se	0.5	0.5	0.6	0.3
Metatarsus (mm)	$\bar{x}$	26.2	26.1	25.7	26.3
	sd	0.9	1.1	1.2	1.2
	se	0.3	0.4	0.3	0.4

x p 0.01
xx p 0.005
xxx p 0.001

HET CODE : BE SUBSTANCE CODE : Lusantan T3

Inj. d 5

Parameter		10.0 mg/egg	mg/egg	mg/egg	mg/egg
Skull diameter (mm)	$\bar{x}$	13.2			
	sd	0.3			
	se	0.1			
Humerus (mm)	$\bar{x}$	18.3			
	sd	0.8			
	se	0.2			
Ulna (mm)	$\bar{x}$	18.4			
	sd	0.8			
	se	0.2			
Femur (mm)	$\bar{x}$	24.0			
	sd	0.6			
	se	0.2			
Tibia (mm)	$\bar{x}$	33.7			
	sd	1.2			
	se	0.3			
Metatarsus (mm)	$\bar{x}$	27.3 **			
	sd	0.6			
	se	0.2			

x p 0.01
xx p 0.005
xxx p 0.001

HET CODE : BE

SUBSTANCE CODE : Lusantan T3

Inj. d 5

Biochemical parameter		0.0 mg/egg	0.25 mg/egg	1.0 mg/egg	2.5 mg/egg
Tot. Bilirubin (mg/dl)	$\bar{x}$				
	sd				
	se				
GOT (U/l (25°))	$\bar{x}$	77.00	79.33	74.50	76.20
	sd	2.92	5.13	6.61	12.95
	se	1.30	2.96	3.30	5.79
GPT (U/l (25°))	$\bar{x}$	11.75	7.00	9.75	8.00
	sd	6.50	2.00	3.86	2.58
	se	3.25	1.15	1.93	1.29
LDH (U/l (25°))	$\bar{x}$	728.60	598.33	749.20	809.40
	sd	178.77	42.15	133.45	160.92
	se	79.95	24.33	59.68	71.97
AP (U/l (25°))	$\bar{x}$	1728.00	1813.33	1736.00	1844.00
	sd	141.84	175.02	297.12	137.95
	se	63.44	101.05	132.88	61.69
γ-GT (U/l (25°))	$\bar{x}$	4.00	4.00	4.20	3.00 *
	sd	0.00	0.00	0.84	0.00
	se	0.00	0.00	0.37	0.00

x p 0.01
xx p 0.005
xxx p 0.001

HET CODE : BE

SUBSTANCE CODE : Lusantan T3

Inj. d 5

Biochemical parameter		10.0 mg/egg	mg/egg	mg/egg	mg/egg
Tot. Bilirubin (mg/dl)	$\bar{x}$ sd se				
GOT (U/l (25°))	$\bar{x}$ sd se	78.60 9.91 4.43			
GPT (U/l (25°))	$\bar{x}$ sd se	8.40 4.04 1.81			
LDH (U/l (25°))	$\bar{x}$ sd se	879.20 104.70 46.82			
AP (U/l (25°))	$\bar{x}$ sd se	1656.00 73.69 32.95			
γ-GT (U/l (25°))	$\bar{x}$ sd se	3.00*** 0.00 0.00			

x p 0.01
xx p 0.005
xxx p 0.001

HET CODE : BE SUBSTANCE CODE : Lusantan T3

Inj. d 5

Biochemical parameter		0.0 mg/egg	0.25 mg/egg	1.0 mg/egg	2.5 mg/egg
Tot. protein (g/dl)	$\bar{x}$	1.54	1.47	1.64	1.78
	sd	0.15	0.35	0.27	0.24
	se	0.07	0.20	0.12	0.11
Creatinine (mg/dl)	$\bar{x}$	0.32	0.30	0.34	0.32
	sd	0.04	0.10	0.05	0.04
	se	0.02	0.06	0.02	0.02
Glucose (mg/dl)	$\bar{x}$	246.00	233.67	233.80	240.20
	sd	16.99	6.66	11.52	10.31
	se	7.60	3.84	5.15	4.61
Uric acid (mg/dl)	$\bar{x}$	5.66	5.30	5.04	3.96
	sd	1.55	0.44	1.66	0.56
	se	0.70	0.25	0.74	0.25
Cholesterol (mg/dl)	$\bar{x}$	374.80	390.33	403.40	377.20
	sd	49.71	66.98	42.54	24.50
	se	22.23	38.67	19.03	10.96
Triglycerides (mg/dl)	$\bar{x}$	133.40	125.00	100.80	88.80***
	sd	14.88	12.12	5.89	8.70
	se	6.65	7.00	2.63	3.89

x p 0.01
xx p 0.005
xxx p 0.001

HET CODE : BE

SUBSTANCE CODE : Lusantan T3

Inj. d 5

Biochemical parameter		10.0 mg/egg	mg/egg	mg/egg	mg/egg
Tot. protein (g/dl)	$\bar{x}$	1.72			
	sd	0.23			
	se	0.10			
Creatinine (mg/dl)	$\bar{x}$	0.34			
	sd	0.50			
	se	0.02			
Glucose (mg/dl)	$\bar{x}$	241.00			
	sd	14.86			
	se	6.57			
Uric acid (mg/dl)	$\bar{x}$	4.26			
	sd	1.64			
	se	0.65			
Cholesterol (mg/dl)	$\bar{x}$	358.00			
	sd	38.87			
	se	17.38			
Triglycerides (mg/dl)	$\bar{x}$	92.20***			
	sd	15.10			
	se	6.71			

x p 0.01
xx p 0.005
xxx p 0.001

HET CODE : BE

SUBSTANCE CODE : Lusantan T3

Inj. d 5

Biochemical parameter		0.0 mg/egg	0.25 mg/egg	1.0 mg/egg	2.5 mg/egg
Sodium (mmol/l)	$\bar{x}$	135.20	134.00	139.40	141.6 **
	sd	2.49	2.65	3.13	2.7
	se	1.11	1.53	1.40	1.2
Potassium (mmol/l)	$\bar{x}$	7.98	5.67	6.82	6.7
	sd	2.15	1.85	1.68	1.3
	se	0.96	1.07	0.75	0.6
Calcium (mmol/l)	$\bar{x}$	3.23	2.46	2.62	2.6
	sd	1.66	0.12	0.08	0.0
	se	0.84	0.07	0.04	0.0
Chloride (mmol/l)	$\bar{x}$	104.40	102.00	105.80	106.6
	sd	2.61	3.61	3.77	3.6
	se	1.17	2.08	1.69	1.6
Inorg. phosphate (mg/dl)	$\bar{x}$	5.12	4.13	5.04	4.7
	sd	0.40	0.15	0.50	0.6
	se	0.18	0.09	0.22	0.2

x p 0.01
xx p 0.005
xxx p 0.001

HET CODE : BE SUBSTANCE CODE : Lusantan T3

Inj. d 5

Biochemical parameter		10.0 mg/egg	mg/egg	mg/egg	mg/egg
Sodium (mmol/l)	$\bar{x}$	142.20***			
	sd	3.35			
	se	1.50			
Potassium (mmol/l)	$\bar{x}$	6.54			
	sd	1.53			
	se	0.68			
Calcium (mmol/l)	$\bar{x}$	2.57			
	sd	0.10			
	se	0.05			
Chloride (mmol/l)	$\bar{x}$	105.80			
	sd	2.86			
	se	1.28			
Inorg. phosphate (mg/dl)	$\bar{x}$	5.00			
	sd	0.90			
	se	0.40			

x p 0.01
xx p 0.005
xxx p 0.001

Normal values for hen's embryo tests (HET)
(Lohmann Selected Leghorn, LSL; White Leghorn)

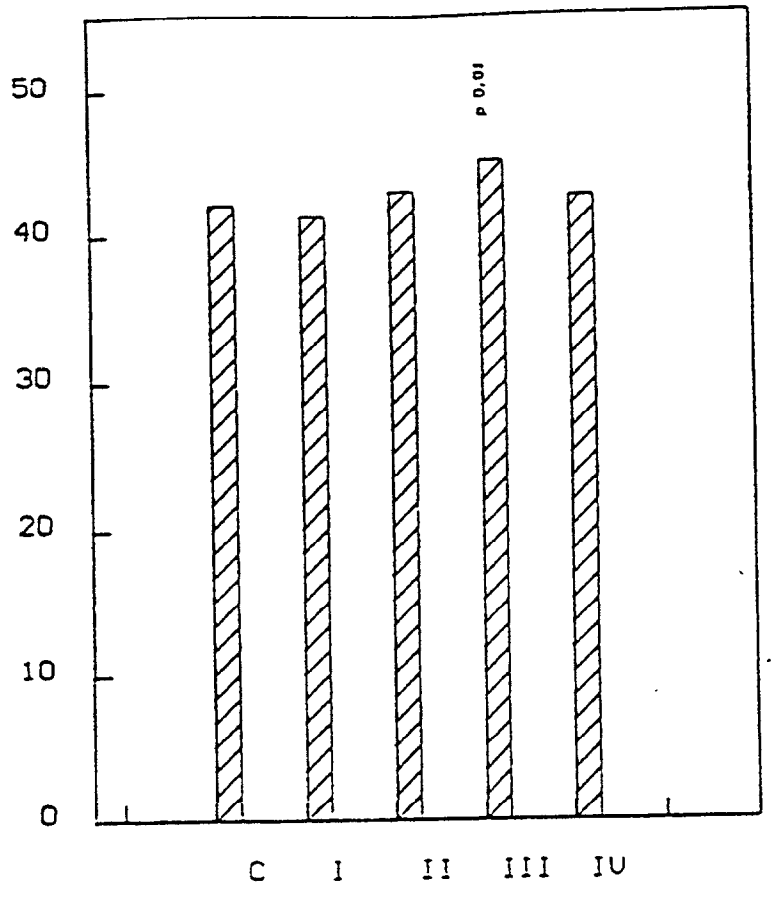
Parameter	Unit	N	$\bar{x}$	s.d.	s.e.
Hatching weight	g	416	41.7	3.6	0.2
Humerus	mm	416	17.2	1.2	0.1
Ulna	mm	416	17.0	1.2	0.1
Femur	mm	416	22.5	1.4	0.1
Tibia	mm	416	33.2	1.6	0.1
Metatarsus	mm	416	25.5	1.6	0.1
Skull thickness	mm	416	13.1	0.3	-
Liver (abs.)	g	416	0.83	0.11	0.01
Liver (rel.)	g/100 g	416	1.98	0.27	0.01
Heart (abs.)	g	416	0.26	0.04	-
Heart (rel.)	g/100 g	416	0.62	0.11	0.01
Sodium	mmol/l	130	136	7.5	0.7
Calcium	mmol/l	140	2.54	0.23	0.02
Chloride	mmol/l	131	106	8.9	0.8
Inorg. phosphate	mg/dl	136	5.0	1.4	0.1
Tot. protein	g/dl	140	1.8	0.4	-
Creatinine	mg/dl	140	0.4	0.1	-
Glucose	mg/dl	132	246	25	2
Uric acid	mg/dl	135	5.7	1.9	0.2
Cholesterol	mg/dl	135	377	60	5
Triglycerides	mg/dl	140	97	37	3
GOT	U/l	123	96	24	2
GPT	U/l	102	12.1	16.2	1.6
LDH	U/l	135	959	264	23
AP	U/l	134	1604	638	55

Table 5

Normal values for hen's embryo tests (HET)
(Shaver Starcross 288A; White Leghorn)

Parameter	Unit	N	$\bar{x}$	s.d.	s.e.
Hatching weight	g	457	41.3	1.4	0.1
Humerus	mm	457	17.8	1.1	0.1
Ulna	mm	457	16.7	1.2	0.1
Femur	mm	457	24.5	1.3	0.1
Tibia	mm	457	34.1	1.3	0.1
Metatarsus	mm	457	25.3	1.1	0.1
Skull thickness	mm	457	13.2	0.2	-
Liver (abs.)	g	457	0.83	0.09	-
Liver (rel.)	g/100 g	457	2.00	0.23	0.01
Heart (abs.)	g	457	0.25	0.02	-
Heart (rel.)	g/100 g	457	0.61	0.06	-
Sodium	mmol/l	165	132	6	0.5
Calcium	mmol/l	167	2.41	0.12	0.01
Chloride	mmol/l	168	104	5	0.4
Inorg. phosphate	mg/dl	165	4.5	0.9	0.1
Tot. protein	g/dl	169	1.9	0.4	-
Creatinine	mg/dl	166	0.4	0.1	-
Glucose	mg/dl	169	219	15	1
Uric acid	mg/dl	167	4.4	1.4	0.1
Cholesterol	mg/dl	151	364	54	4
Triglycerides	mg/dl	167	81	22	2
GOT	U/l	166	91	16	1
GPT	U/l	159	6.5	5.9	0.5
LDH	U/l	104	917	240	24
AP	U/l	167	4273	933	72

Table 5

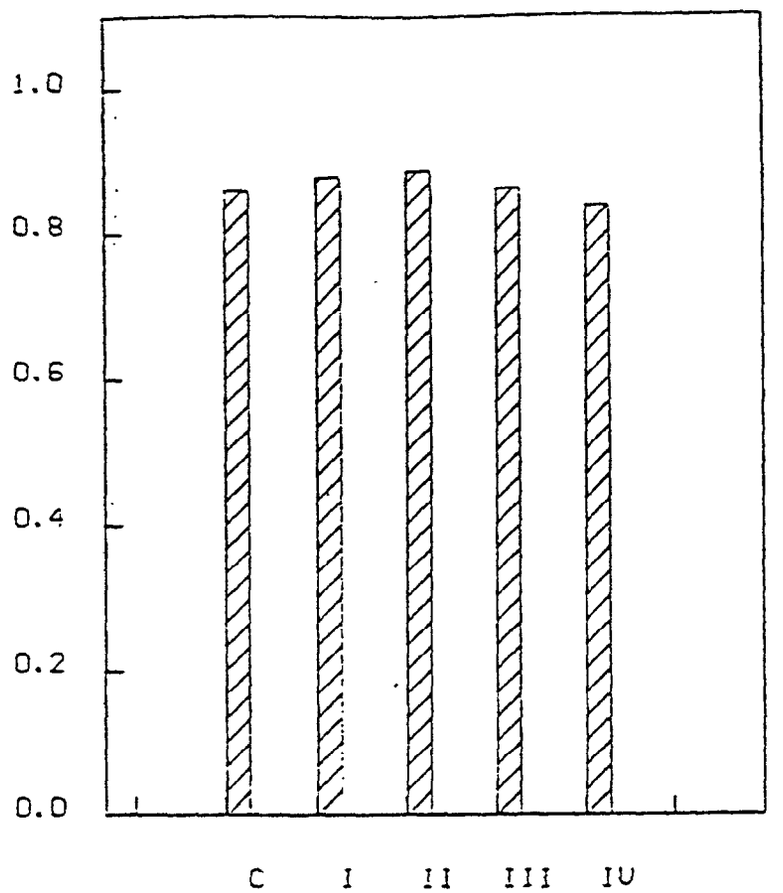


Hatching weight (g)

C	Control	0 mg/egg
I	1.0	mg/egg
II	2.5	mg/egg
III	10.0	mg/egg
IV	25.0	mg/egg

Lusantan T 3

d1

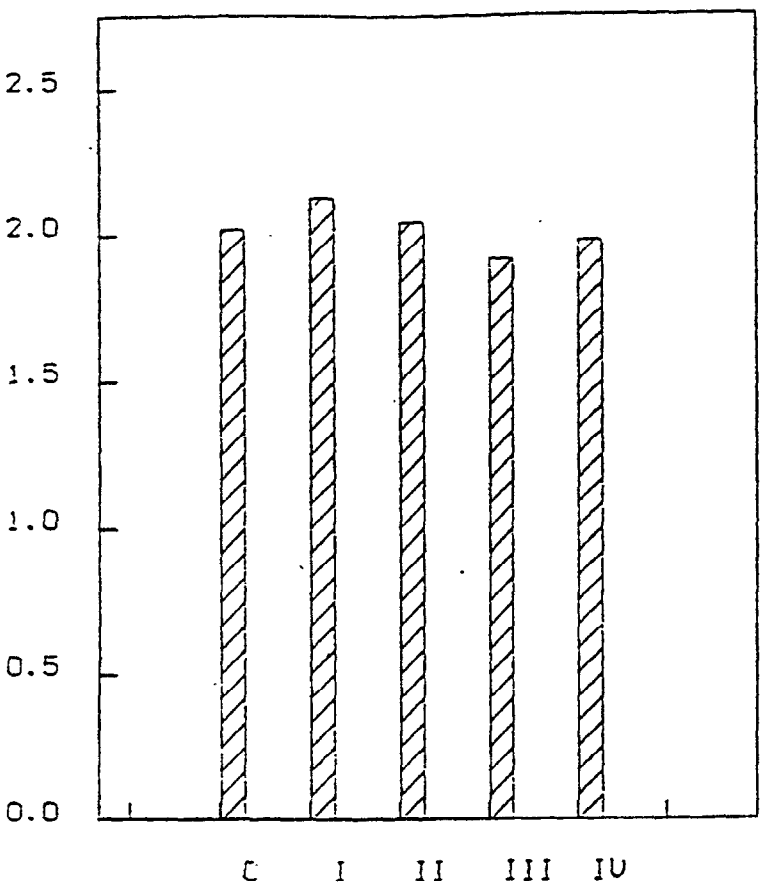


Liver weight abs. (g)

C	Control	0 mg/egg
I	1.0	mg/egg
II	2.5	mg/egg
III	10.0	mg/egg
IV	25.0	mg/egg

Lusantan T 3

d1

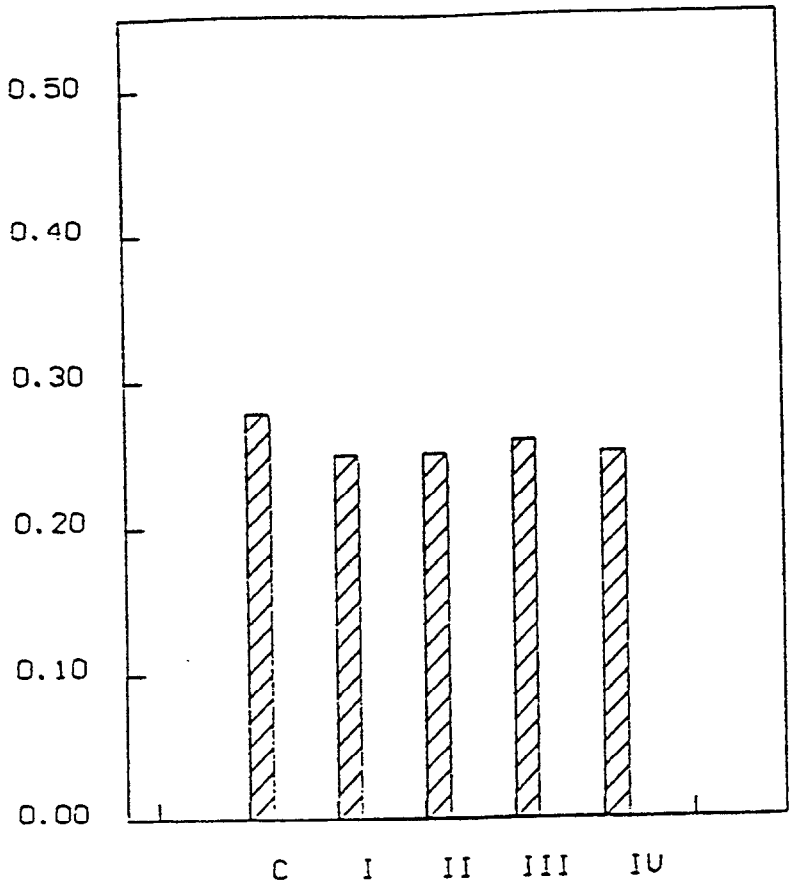


Liver weight rel. (g/100 g)

C	Control	0 mg/egg
I	1.0	mg/egg
II	2.5	mg/egg
III	10.0	mg/egg
IV	25.0	mg/egg

Lusantan T 3

dl

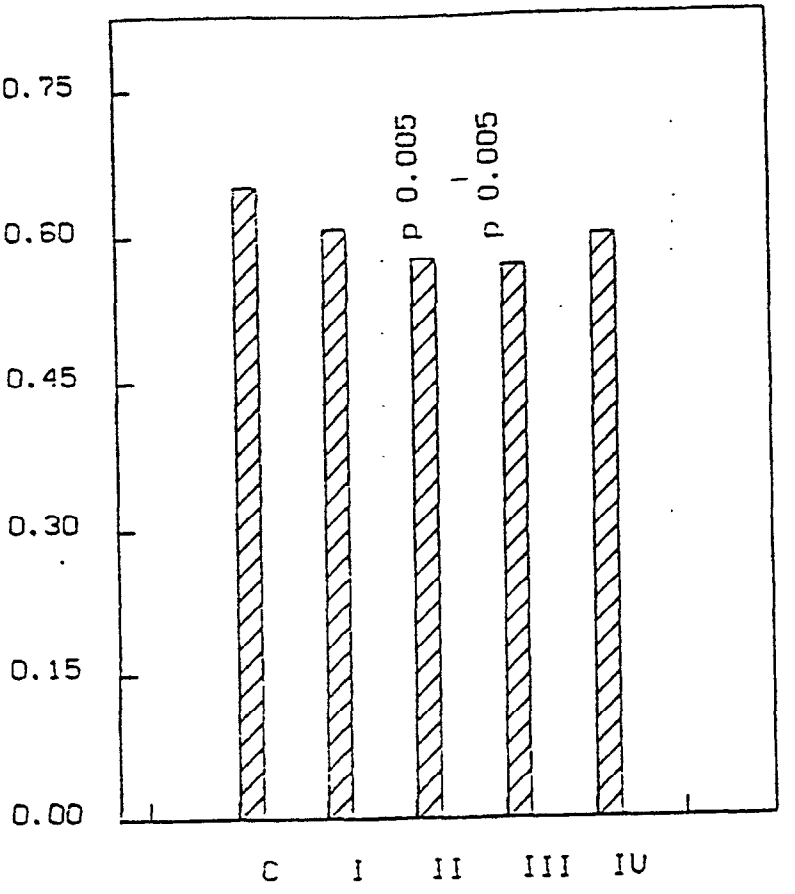


Heart weight abs. (g)

C	Control	0 mg/egg
I	1.0	mg/egg
II	2.5	mg/egg
III	10.0	mg/egg
IV	25.0	mg/egg

Lusantan T 3

d1

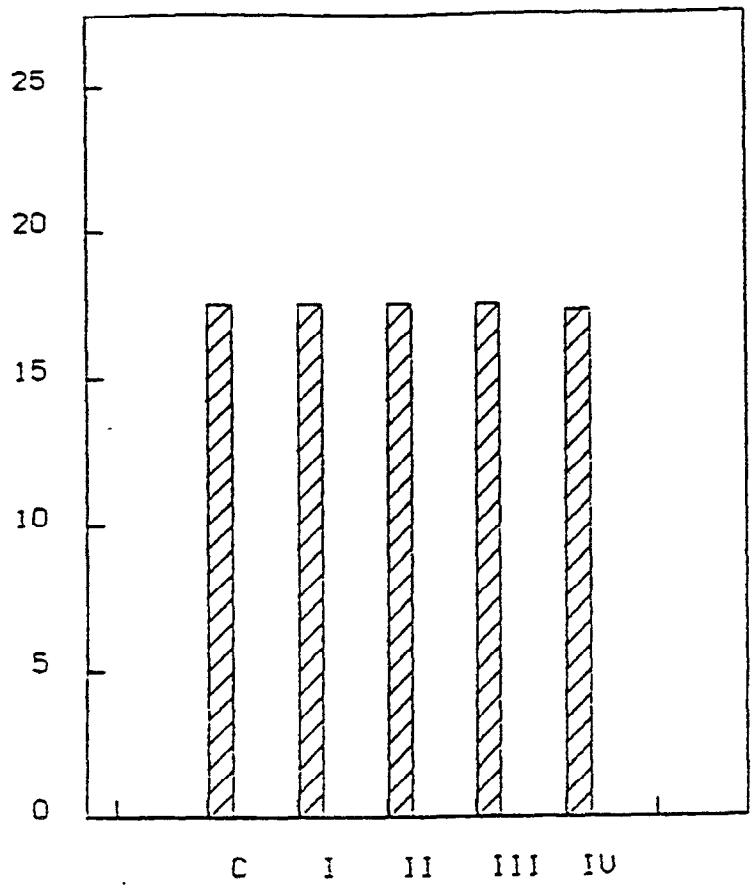


Heart weight rel. (g/100 g)

C	Control	0 mg/egg
I	1.0	mg/egg
II	2.5	mg/egg
III	10.0	mg/egg
IV	25.0	mg/egg

Lusantan T 3

d 1

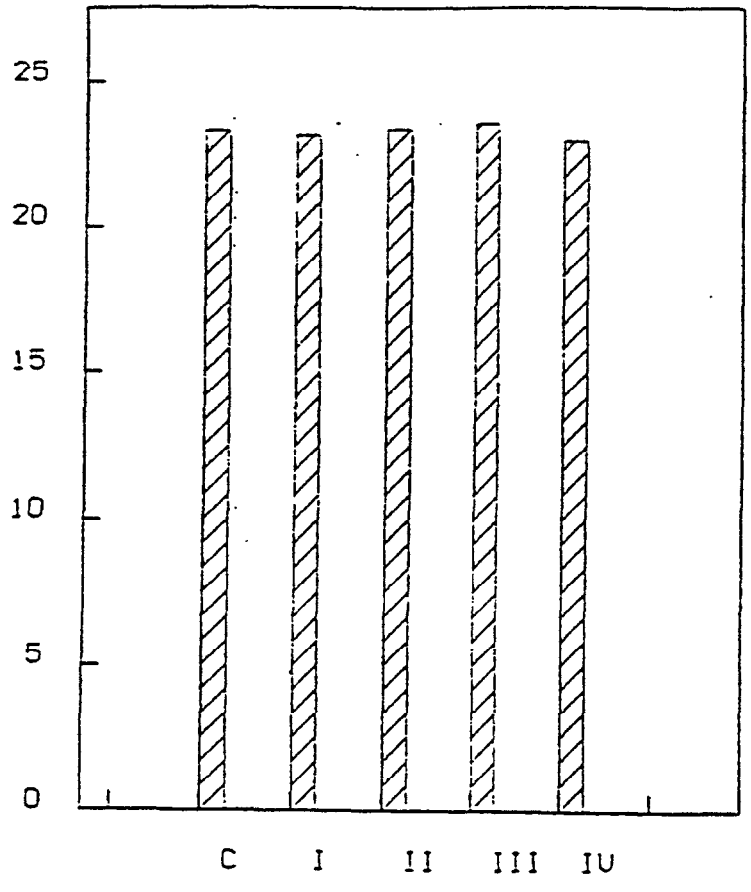


Humerus (mm)

C	Control	0 mg/egg
I	1.0	mg/egg
II	2.5	mg/egg
III	10.0	mg/egg
IV	25.0	mg/egg

Lusantan T 3

d 1

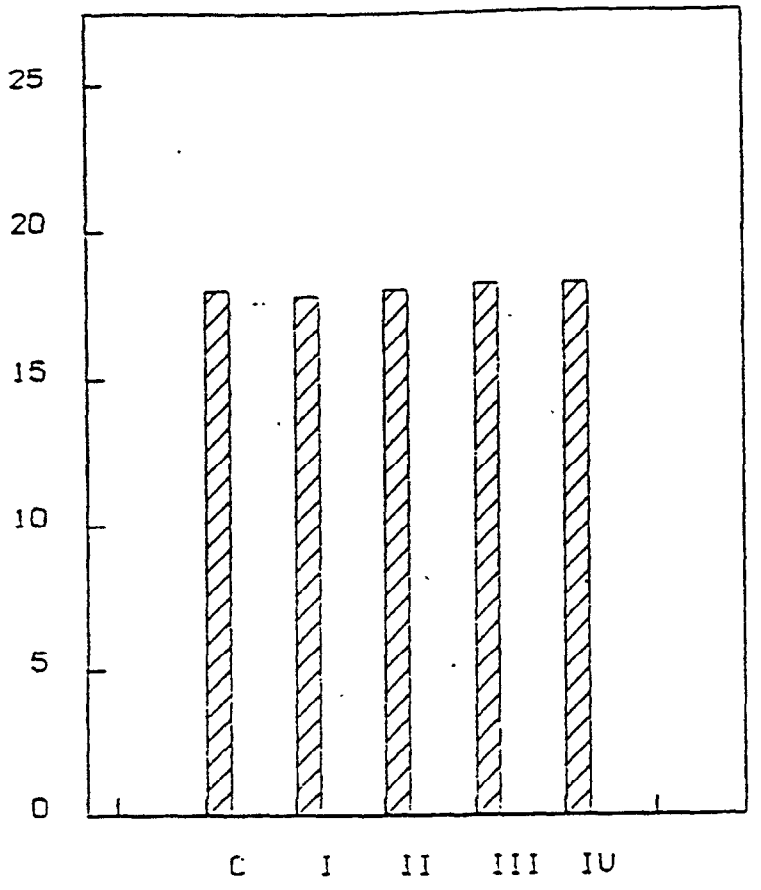


FEMUR (mm)

C	CONTROL	0 mg/egg
I	1.0	mg/egg
II	2.5	mg/egg
III	10.0	mg/egg
IV	25.0	mg/egg

Lusantan T 3

d 1

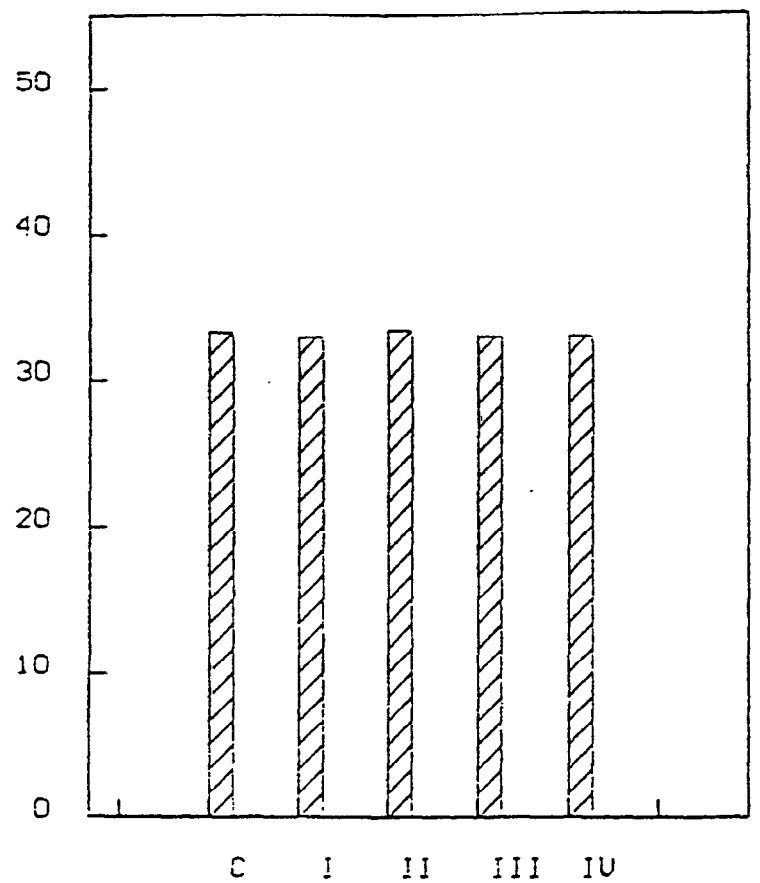


ULNA (mm)

C	Control	0 mg/egg
I	1.0	mg/egg
II	2.5	mg/egg
III	10.0	mg/egg
IV	25.0	mg/egg

d 1

Lusantan T 3

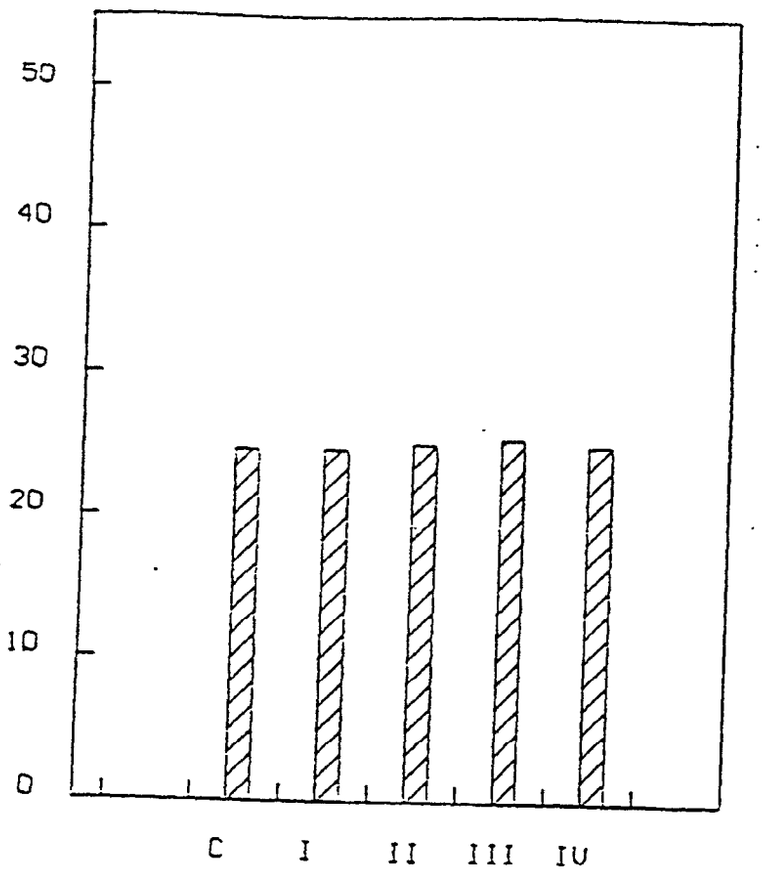


TIBIA (mm)

C	CONTROL	0 mg/egg
I	1.0	mg/egg
II	2.5	mg/egg
III	10.0	mg/egg
IV	25.0	mg/egg

d 1

Lusantan T 3

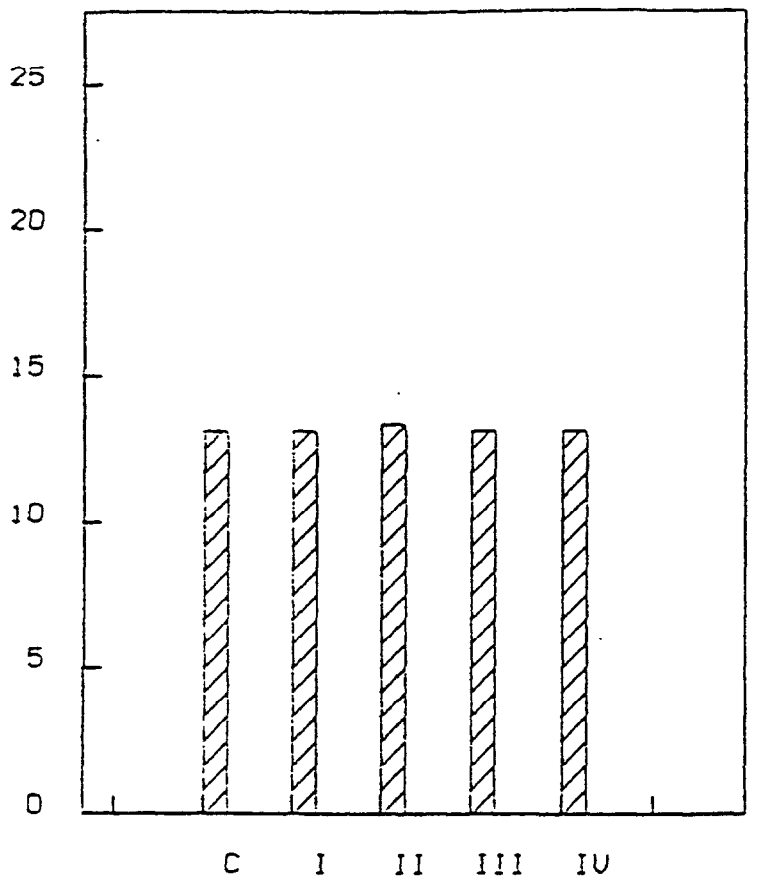


METATARSUS (mm)

C	CONTROL	0 mg/egg
I	1.0	mg/egg
II	2.5	mg/egg
III	10.0	mg/egg
IV	25.0	mg/egg

d 1

Lusantan T 3

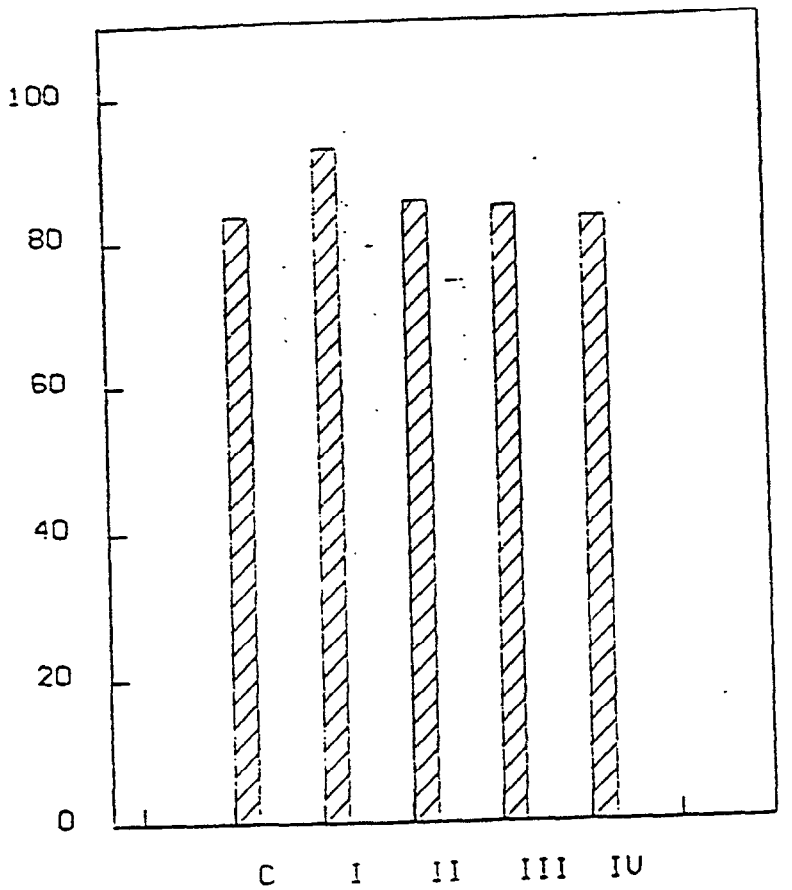


Skull diameter (mm)

C	Control	0 mg/egg
I	1.0	mg/egg
II	2.5	mg/egg
III	10.0	mg/egg
IV	25.0	mg/egg

Lusantan T 3

d 1

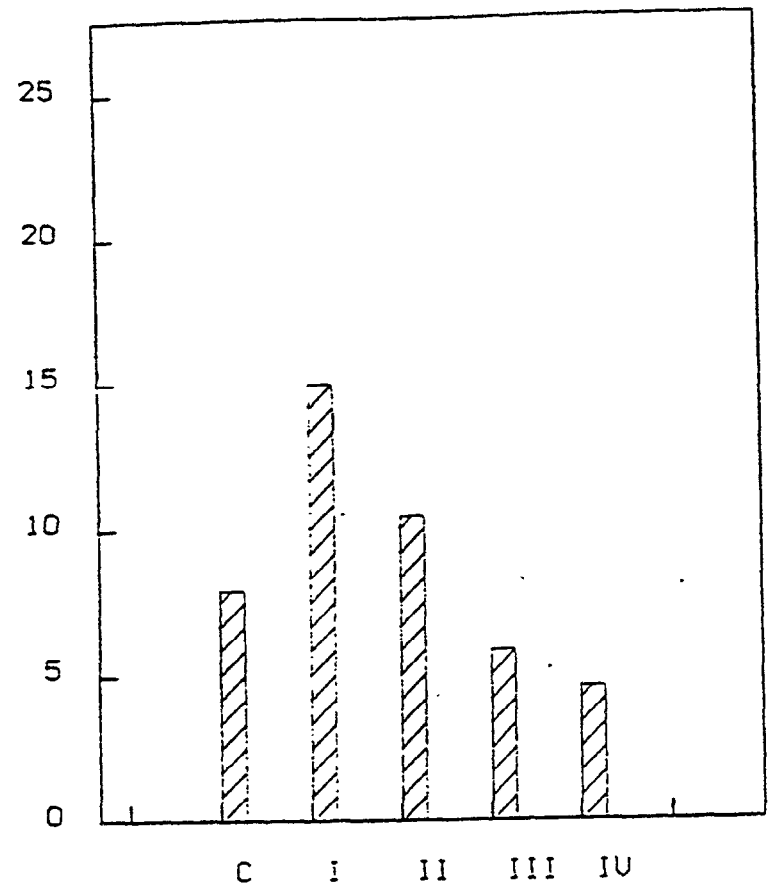


GOT (U/l)

C	CONTROL	0 mg/egg
I	1.0	mg/egg
II	2.5	mg/egg
III	10.0	mg/egg
IV	25.0	mg/egg

Lusantan T 3

d 1

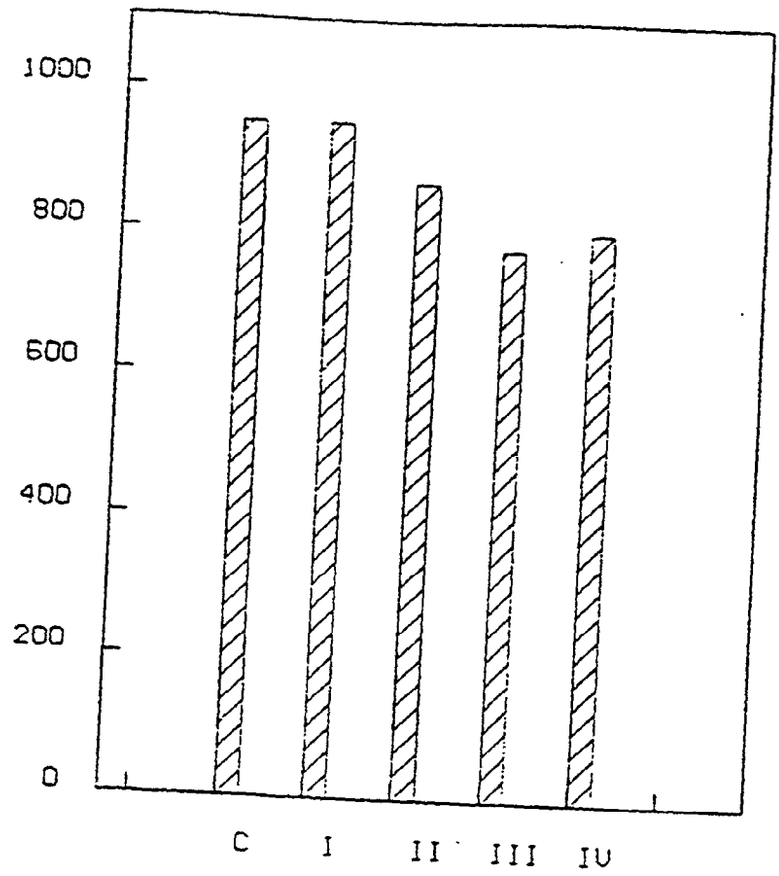


GPT (U/l)

C	CONTROL	0 mg/egg
I	1.0	mg/egg
II	2.5	mg/egg
III	10.0	mg/egg
IV	25.0	mg/egg

Lusantan T 3

d 1

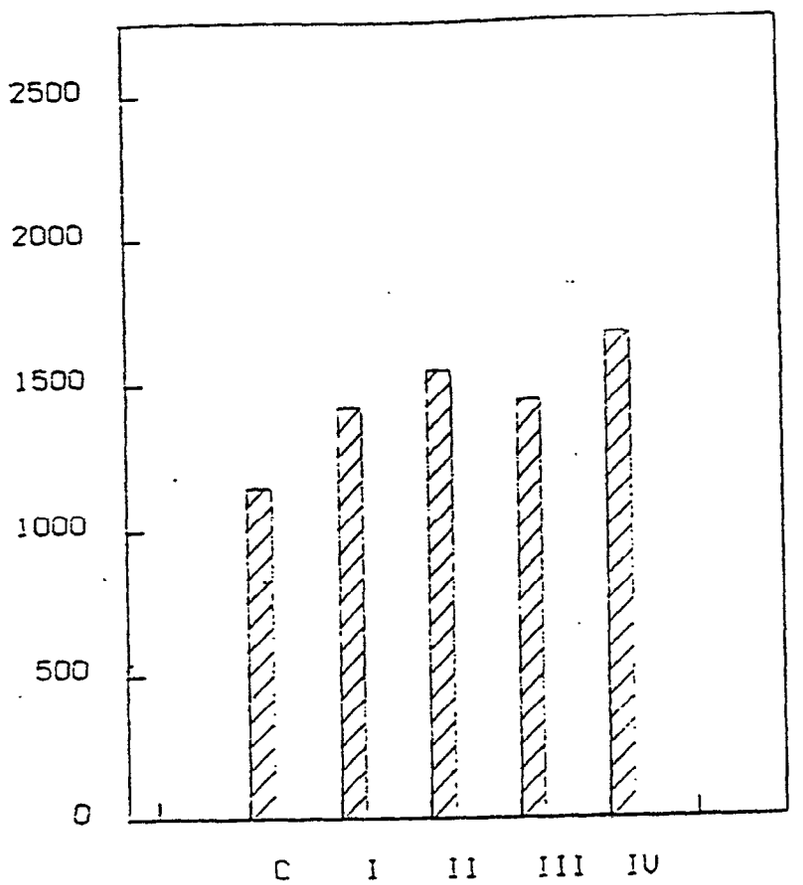


LDH (U/l)

C	CONTROL	0 mg/egg
I	1.0	mg/egg
II	2.5	mg/egg
III	10.0	mg/egg
IV	25.0	mg/egg

d 1

Lusantan T 3

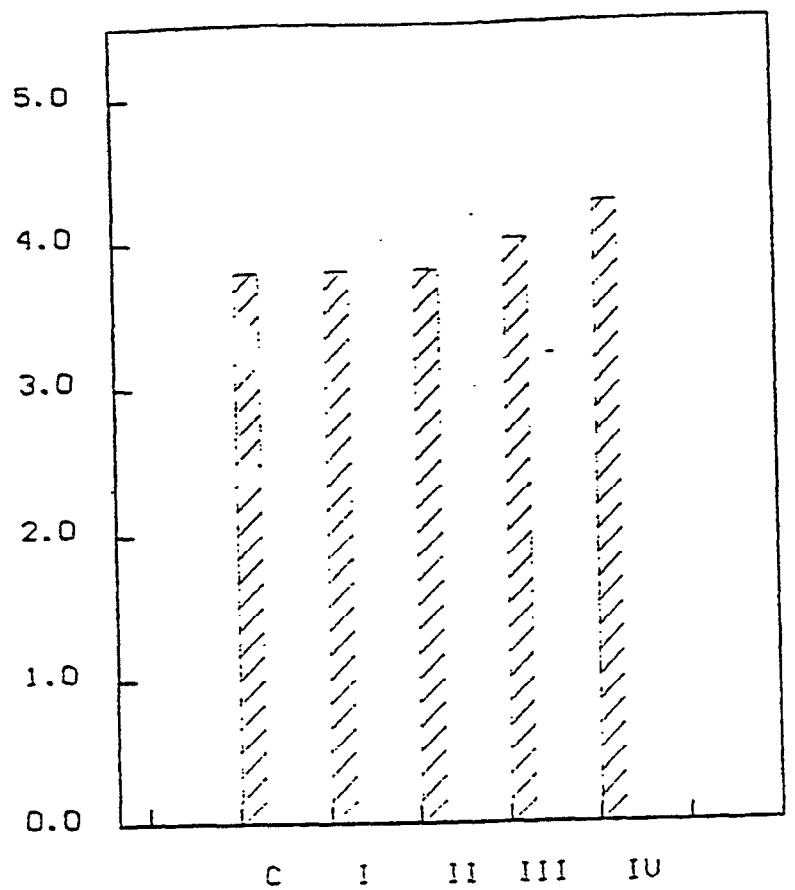


AP (U/l)

C	CONTROL	0 mg/egg
I	1.0	mg/egg
II	2.5	mg/egg
III	10.0	mg/egg
IV	25.0	mg/egg

Lusantan T 3

d 1

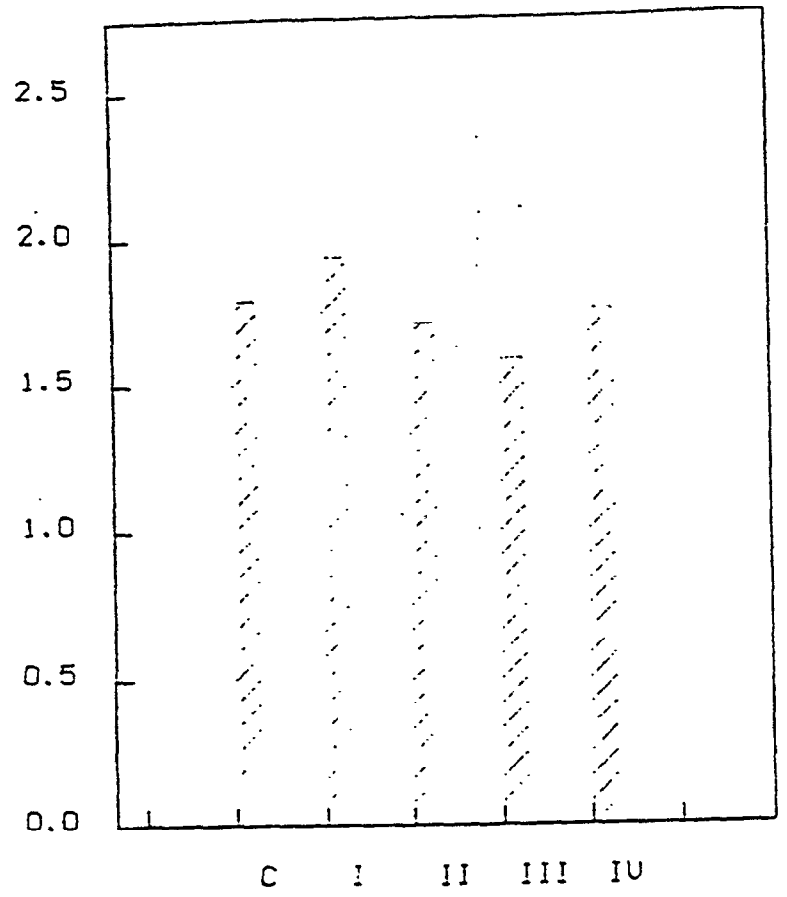


gamma-GT (U/l)

C	CONTROL	0 mg/egg
I	1.0	mg/egg
II	2.5	mg/egg
III	10.0	mg/egg
IV	25.0	mg/egg

d 1

Lusantan T 3

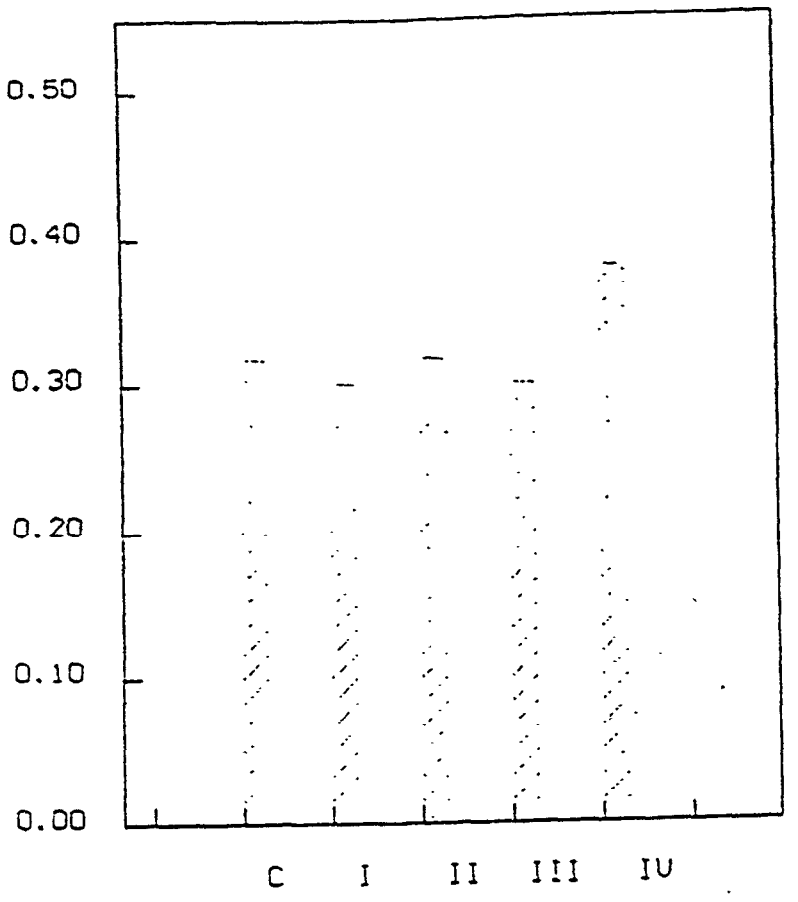


TOT. PROTEIN (g/dl)

C	CONTROL	0 mg/egg
I	1.0	mg/egg
II	2.5	mg/egg
III	10.0	mg/egg
IV	25.0	mg/egg

d 1

Lusantan T 3

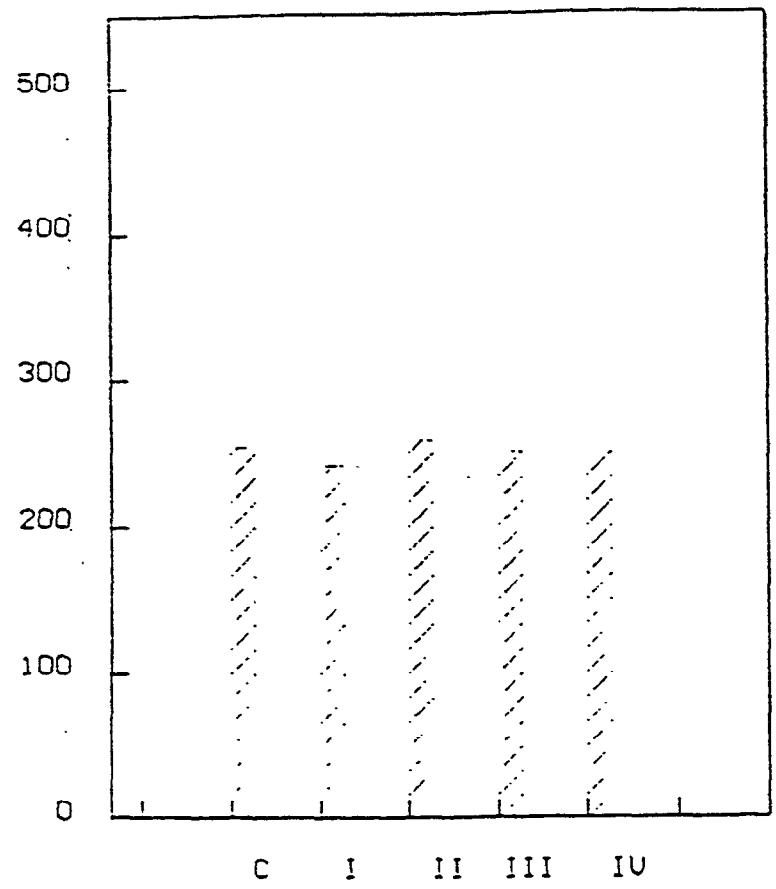


CREATININE (mg/dl)

C	CONTROL	0 mg/egg
I	1.0	mg/egg
II	2.5	mg/egg
III	10.0	mg/egg
IV	25.0	mg/egg

d 1

Lusantan T 3

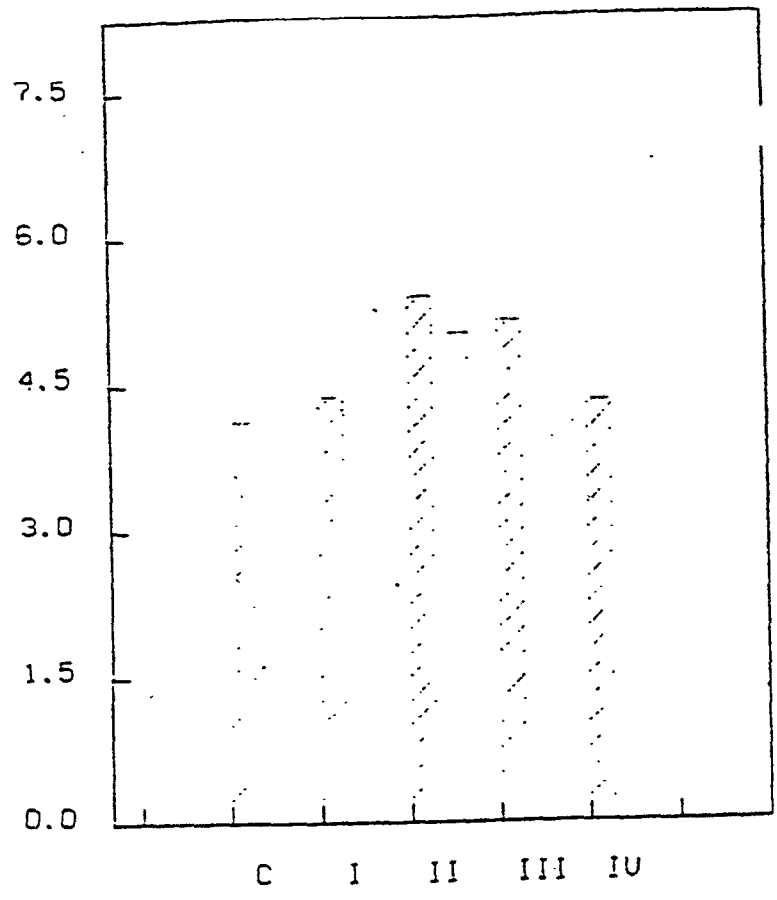


GLUCOSE (mg/dl)

C	CONTROL	0 mg/egg
I	1.0	mg/egg
II	2.5	mg/egg
III	10.0	mg/egg
IV	25.0	mg/egg

d 1

Lusantan T 3

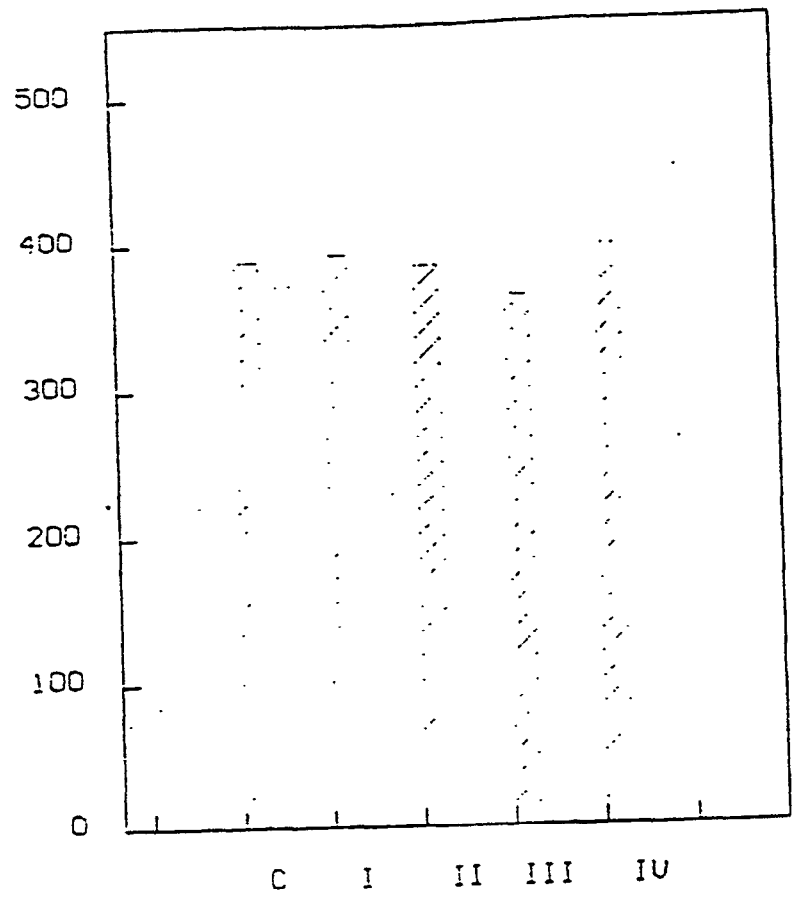


URIC ACID (mg/dl)

C	CONTROL	0 mg/egg
I	1.0	mg/egg
II	2.5	mg/egg
III	10.0	mg/egg
IV	25.0	mg/egg

Lusantan T 3

d 1

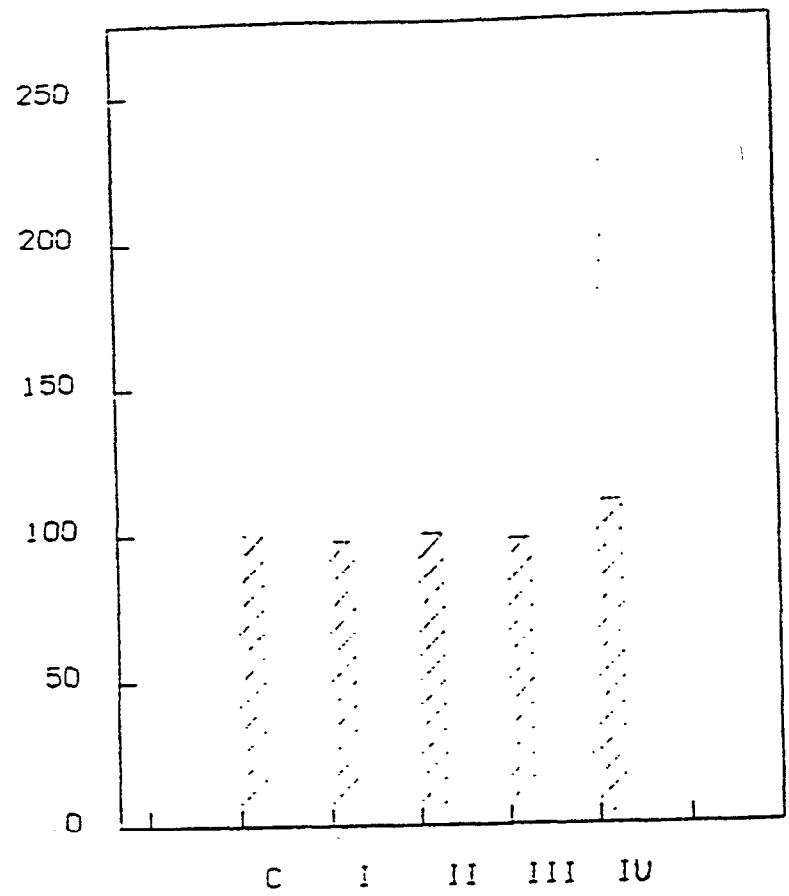


CHOLESTEROL (mg/dl)

C	CONTROL	0 mg/egg
I	1.0	mg/egg
II	2.5	mg/egg
III	10.0	mg/egg
IV	25.0	mg/egg

d 1

Lusantan T 3

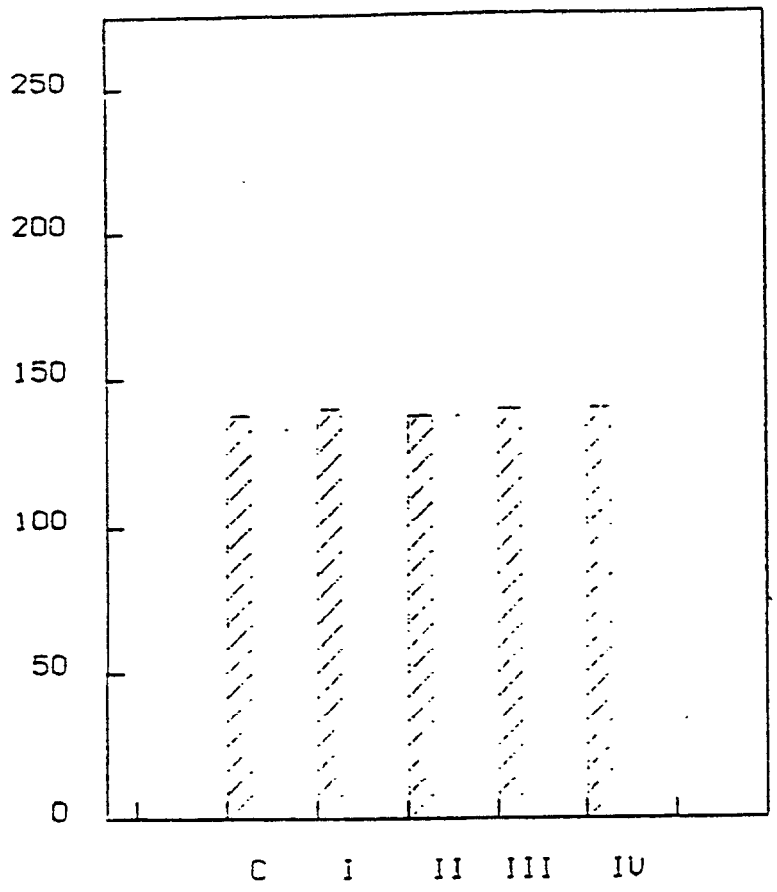


TRIGLYCERIDES (mg/dl)

C	CONTROL	0	mg/egg
I	1.0		mg/egg
II	2.5		mg/egg
III	10.0		mg/egg
IV	25.0		mg/egg

Lusantan T 3

d 1

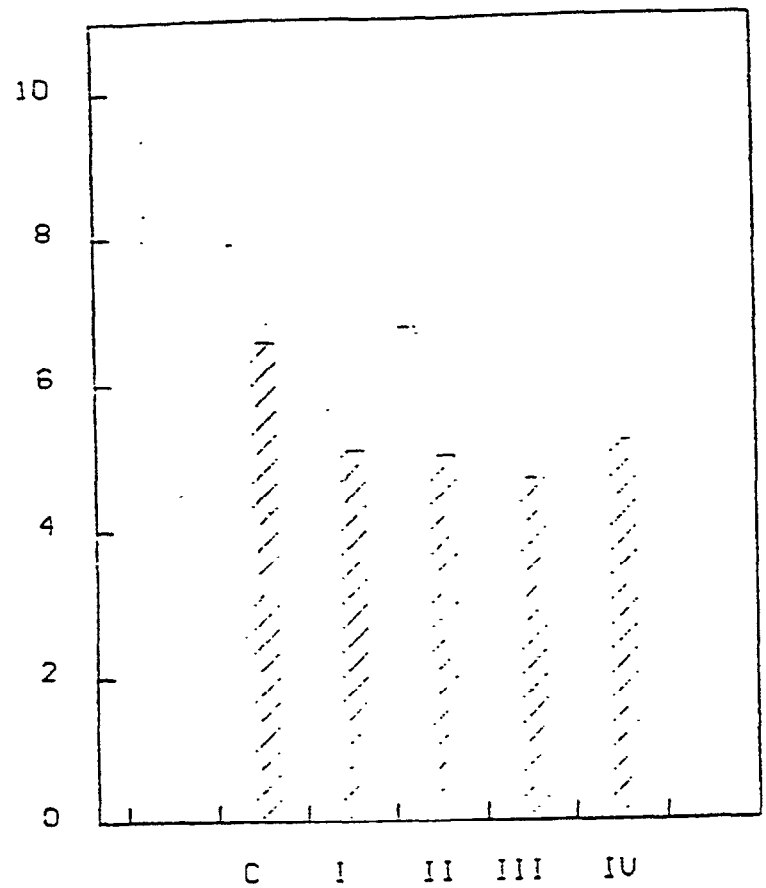


SODIUM (mmol/l)

C	CONTROL 0	mg/egg
I	1.0	mg/egg
II	2.5	mg/egg
III	10.0 (2.5)	mg/egg
IV	25.0	mg/egg

d 1

Lusantan T 3

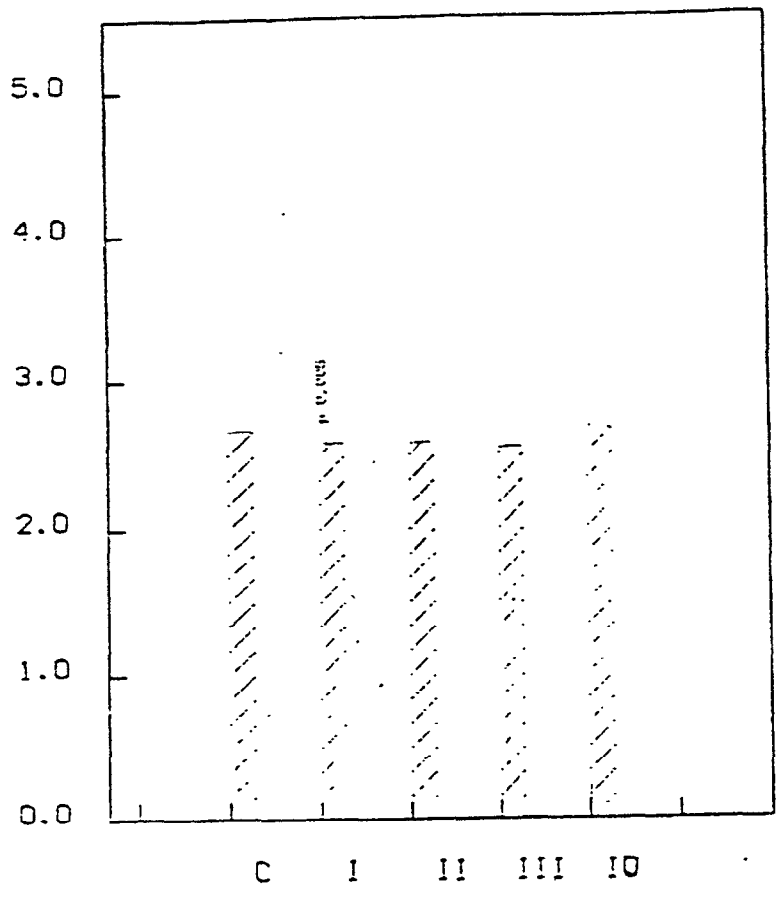


POTASSIUM (mmol/l)

C	CONTROL	0 mg/egg
I	1.0	mg/egg
II	2.5	mg/egg
III	10.0	mg/egg
IV	25.0	mg/egg

Lusantan T 3

d 1

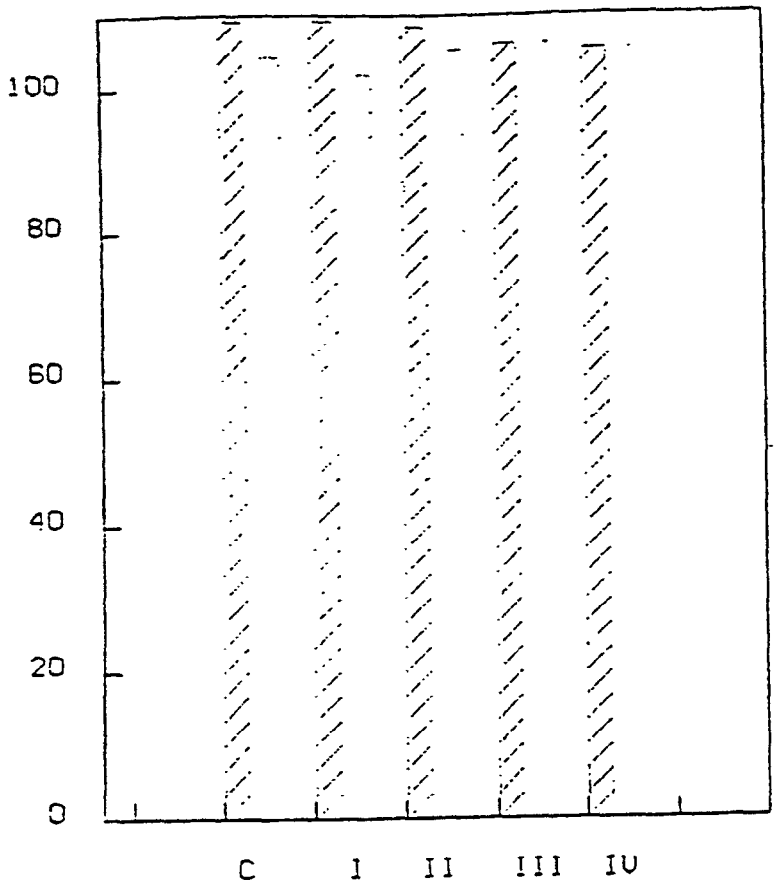


CALCIUM (mmol/l)

C	CONTROL	0 mg/egg
I	1.0	mg/egg
II	2.5	mg/egg
III	10.0	mg/egg
IV	25.0	mg/egg

Lusantan T 3

d 1

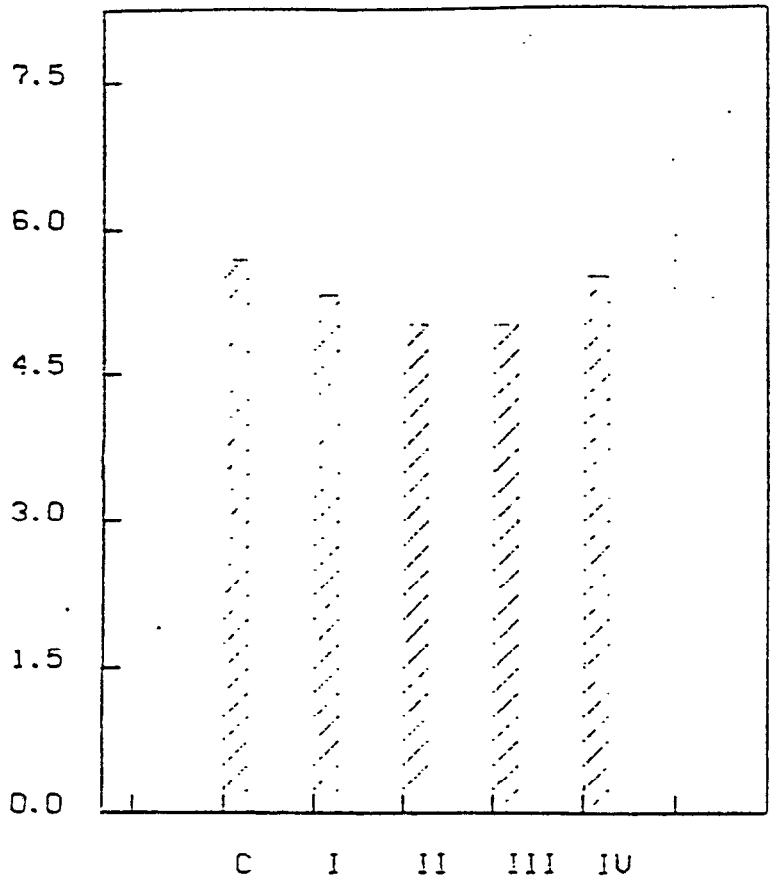


CHLORIDE (mmol/l)

C	CONTROL	0 mg/egg
I	1.0	mg/egg
II	2.5	mg/egg
III	10.0	mg/egg
IV	25.0	mg/egg

Lusantan T 3

d 1



INORG. PHOSPHATE (mg/dl)

C	CONTROL	0 mg/egg
I	1.0	mg/egg
II	2.5	mg/egg
III	10.0	mg/egg
IV	25.0	mg/egg

Lusantan T 3

d 1

TRANSLATION

PROF. DR. N.P. LÜPKE
Institute of Pharmacology and Toxicology
Westphalia University, Münster

D - 4400 MÜNSTER, Domagkstr. 12
Tel. 0251 - 835525

REPORT
ON
TOXICITY TESTS
ON
INCUBATED CHICKEN EGGS

(HET CHORIOALLANTOIC TEST)

TEST SUBSTANCE: LUSANTAN T3
BEIERSDORF AG

N.P. LUEPKE

PROF. DR. N.P. LÜPKE
Institute of Pharmacology and Toxicology
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23.IX.1985

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TEST SUBSTANCE: LUSANTAN T 3
BEIERSDORF AG

The contents of the report are intended only for internal use by Beiersdorf Aktiengesellschaft, D-2000 Hamburg 20, and for submission to the authorities. They may not be used, even in part, for general information or promotional purposes without express approval by the undersigned.

[signed]
Prof. N.P. Luepke

1. SUMMARY

1. SUMMARY

The test substance Lusantan T3 was dissolved or suspended in olive oil and administered in a volume of 0.2 ml/egg to test, in a comparative investigation with olive oil as control, the membrane tolerability on the vital, vascularized, pain-insensitive chorioallantoic membrane of d10-incubated hen's eggs (White Leghorn; Lohmann Selected Leghorn, LSL).

The test concentrations (1 and 10 %) of Lusantan T3 showed the HET CAM categorization

"practically non-irritant".

2. TEST CONDITIONS

2. TEST CONDITIONS

2.1. Test substance

The test substance was provided under the name Lusantan T3 by Beiersdorf AG, D-2000 Hamburg 20, for toxicity tests on incubated chicken eggs (hen's egg test, HET) in an amount of about 50 g in an uncolored wide-necked glass bottle with screw cap and the above labelling.

The test substance was a white, caking powder of melting point 100 - 101°C; batch No. 17745/104. No other physical, chemical or physicochemical data were provided.

According to Beiersdorf AG, Lusantan T3 is a "solid solution" of 2,4,6-tris(p-2-ethylhexoxycarbonylanilino)-s-triazine (Mol. wt. 823) and 2-ethylhexanoic acid C16/C18 ester in the ratio 63.5 % triazine to 36.5 % ester, which is intended to be used as sunscreen agent (UV filter) in cosmetic compositions.

2.2. Livestock material

All the investigations were carried out on fresh hatchery eggs laid by hybrids of the white leghorn breed of the same, genetically controlled breeding strain (Lohmann Selected Leghorn, LSL), which were obtained on the date of laying from a commercial breeding unit (J. Brinkschulte, Gut Aversfeld, Senden/Westf.). Only eggs weighing 50 - 60 g were used. The eggs were stored with the pointed end downwards in a cooled room for 24 hours so that the air bubble was positioned under the round end of the egg, with the yolk and the sensitive embryo underneath. Before the start of the test, the eggs were candled, and eggs with defective shells were eliminated.

2.3. Administration method and incubation

The test substances are dissolved or suspended in an appropriate vehicle under aseptic conditions on a clean bench.

The eggs are incubated (with the round end upwards) in a Schuhmacher incubation unit with continuous circulation of air at 37.5°C (± 1.0°C) and a relative humidity of 70 % (± 10 %) for 10 days; this corresponds to optimized incubation conditions. The eggs are turned automatically every two hours. From day 5 of incubation onward, the eggs are candled with a mercury vapor lamp at intervals of one or two days. Disturbed development or death of the embryos can be detected early by candling, in particular from the condition of the blood vessels and the movements of the embryo in the amnion.

On day 10 of incubation, the eggshell is initially cut around the air space using a dental rotating saw blade and then dissected off. The inner egg membrane is cautiously removed to expose the vascularized, pain-insensitive chorioallantoic membrane. The test substance is administered in a volume of 0.2 ml of solution or suspension; in the case of solid test substances, 0.1 g is applied to the membrane and rinsed off with 5 ml of warm water after 20 seconds. 4-6 eggs are tested per test substance concentration; at least 4 other membranes are used as vehicle control. After application of the test substance, the chorioallantoic membrane, the blood vessels including the capillary network and the egg white are checked and assessed for irritant effects (vascular injection, hemorrhages, coagulation) 0.5, 2 and 5 minutes after application. The total score for the three times is calculated. The average from at least 4 tests permits classification in analogy to the Draize scheme (Tab. 1).

3. TEST RESULTS

3. Test results

The following tables show the individual and total scores for the test substance in the toxicity test on incubated hen's eggs (HET chorioallantoic test).

HET CODE: LT 3 EGG No.: 1 SUBST. CODE: LT 3
APPL D: 10 APPL VOL: 0.2 ml CONCENTRATION: 0 %
VEHICLE: Olive oil

TIME (MIN)

EFFECT	.5	2	5	SCORE
INJECTION	-	-	+	1
HEMORRHAGE	-	-	-	-
COAGULATION	-	-	-	-
			SA.:	1

HET CODE: LT 3 EGG No.: 2 SUBST. CODE: LT 3
APPL D: 10 APPL VOL: 0.2 ml CONCENTRATION: 0 %
VEHICLE: Olive oil

TIME (MIN)

EFFECT	.5	2	5	SCORE
INJECTION	-	-	-	-
HEMORRHAGE	-	-	-	-
COAGULATION	-	-	-	-
			SA.:	0

HET CODE: LT 3 EGG No.: 3 SUBST. CODE: LT 3
APPL D: 10 APPL VOL: 0.2 ml CONCENTRATION: 0 %
VEHICLE: Olive oil

TIME (MIN)

EFFECT	.5	2	5	SCORE
INJECTION	-	-	-	-
HEMORRHAGE	-	-	-	-
COAGULATION	-	-	-	-
			SA.:	0

HET CODE: LT 3 EGG No.: 4 SUBST. CODE: LT 3
APPL D: 10 APPL VOL: 0.2 ml CONCENTRATION: 0 %
VEHICLE: Olive oil

TIME (MIN)

EFFECT	.5	2	5	SCORE
INJECTION	-	-	-	-
HEMORRHAGE	-	-	-	-
COAGULATION	-	-	-	-
			SA.:	0

HET CODE: LT 3 EGG No.: 5 SUBST. CODE: LT 3
APPL D: 10 APPL VOL: 0.2 ml CONCENTRATION: 0 %
VEHICLE: Olive oil

TIME (MIN)

EFFECT	.5	2	5	SCORE
INJECTION	-	-	+	1
HEMORRHAGE	-	-	-	-
COAGULATION	-	-	-	-

SA.: 1

HET CODE: LT 3 EGG No.: 6 SUBST. CODE: LT 3
APPL D: 10 APPL VOL: 0.2 ml CONCENTRATION: 0 %
VEHICLE: Olive oil

TIME (MIN)

EFFECT	.5	2	5	SCORE
INJECTION	-	-	-	-
HEMORRHAGE	-	-	-	-
COAGULATION	-	-	-	-

SA.: 0

HET CODE: EGG No.: SUBST. CODE:
APPL D: 10 APPL VOL: 0.2 ml CONCENTRATION:
VEHICLE:

TIME (MIN)

EFFECT	.5	2	5	SCORE
INJECTION				
HEMORRHAGE				
COAGULATION				

SA.:

HET CODE: EGG No.: SUBST. CODE:
APPL D: 10 APPL VOL: 0.2 ml CONCENTRATION:
VEHICLE:

TIME (MIN)

EFFECT	.5	2	5	SCORE
INJECTION				
HEMORRHAGE				
COAGULATION				

SA.:

HET CODE: LT 3 EGG No.: 7 SUBST. CODE: LT 3
APPL D: 10 APPL VOL: 0.2 ml CONCENTRATION: 10 %
VEHICLE: Olive oil

TIME (MIN)

EFFECT	.5	2	5	SCORE
INJECTION	-	-	-	-
HEMORRHAGE	-	-	-	-
COAGULATION	-	-	-	-
			SA.:	0

HET CODE: LT 3 EGG No.: 8 SUBST. CODE: LT 3
APPL D: 10 APPL VOL: 0.2 ml CONCENTRATION: 10 %
VEHICLE: Olive oil

TIME (MIN)

EFFECT	.5	2	5	SCORE
INJECTION	-	-	+	1
HEMORRHAGE	-	-	-	-
COAGULATION	-	-	-	-
			SA.:	1

HET CODE: LT 3 EGG No.: 9 SUBST. CODE: LT 3
APPL D: 10 APPL VOL: 0.2 ml CONCENTRATION: 10 %
VEHICLE: Olive oil

TIME (MIN)

EFFECT	.5	2	5	SCORE
INJECTION	-	-	+	1
HEMORRHAGE	-	-	-	-
COAGULATION	-	-	-	-
			SA.:	1

HET CODE: LT 3 EGG No.: 10 SUBST. CODE: LT 3
APPL D: 10 APPL VOL: 0.2 ml CONCENTRATION: 10 %
VEHICLE: Olive oil

TIME (MIN)

EFFECT	.5	2	5	SCORE
INJECTION	-	-	-	-
HEMORRHAGE	-	-	-	-
COAGULATION	-	-	-	-
			SA.:	0

HET CODE: LT 3 EGG No.: 11 SUBST. CODE: LT 3
APPL D: 10 APPL VOL: 0.2 ml CONCENTRATION: 10 %
VEHICLE: Olive oil

TIME (MIN)

EFFECT	.5	2	5	SCORE
INJECTION	-	-	-	-
HEMORRHAGE	-	-	-	-
COAGULATION	-	-	-	-

SA.: 0

HET CODE: LT 3 EGG No.: 12 SUBST. CODE: LT 3
APPL D: 10 APPL VOL: 0.2 ml CONCENTRATION: 10 %
VEHICLE: Olive oil

TIME (MIN)

EFFECT	.5	2	5	SCORE
INJECTION	-	-	-	-
HEMORRHAGE	-	-	-	-
COAGULATION	-	-	-	-

SA.: 0

HET CODE: EGG No.: SUBST. CODE:
APPL D: 10 APPL VOL: 0.2 ml CONCENTRATION:
VEHICLE:

TIME (MIN)

EFFECT	.5	2	5	SCORE
INJECTION				
HEMORRHAGE				
COAGULATION				

SA.:

HET CODE: EGG No.: SUBST. CODE:
APPL D: 10 APPL VOL: 0.2 ml CONCENTRATION:
VEHICLE:

TIME (MIN)

EFFECT	.5	2	5	SCORE
INJECTION				
HEMORRHAGE				
COAGULATION				

SA.: 0

HET CODE: LT 3 EGG No.: 13 SUBST. CODE: LT 3
APPL D: 10 APPL VOL: 0.2 ml CONCENTRATION: 1 %
VEHICLE: Olive oil

TIME (MIN)

EFFECT	.5	2	5	SCORE
INJECTION	-	-	-	-
HEMORRHAGE	-	-	-	-
COAGULATION	-	-	-	-
			SA.:	0

HET CODE: LT 3 EGG No.: 14 SUBST. CODE: LT 3
APPL D: 10 APPL VOL: 0.2 ml CONCENTRATION: 1 %
VEHICLE: Olive oil

TIME (MIN)

EFFECT	.5	2	5	SCORE
INJECTION	-	-	-	-
HEMORRHAGE	-	-	-	-
COAGULATION	-	-	-	-
			SA.:	0

HET CODE: LT 3 EGG No.: 15 SUBST. CODE: LT 3
APPL D: 10 APPL VOL: 0.2 ml CONCENTRATION: 1 %
VEHICLE: Olive oil

TIME (MIN)

EFFECT	.5	2	5	SCORE
INJECTION	-	-	+	1
HEMORRHAGE	-	-	-	-
COAGULATION	-	-	-	-
			SA.:	1

HET CODE: LT 3 EGG No.: 16 SUBST. CODE: LT 3
APPL D: 10 APPL VOL: 0.2 ml CONCENTRATION: 1 %
VEHICLE: Olive oil

TIME (MIN)

EFFECT	.5	2	5	SCORE
INJECTION	-	-	+	1
HEMORRHAGE	-	-	-	-
COAGULATION	-	-	-	-
			SA.:	1

HET CODE: EGG No.: 17 SUBST. CODE: LT 3
APPL D: 10 APPL VOL: 0.2 ml CONCENTRATION: 1 %
VEHICLE: Olive oil

TIME (MIN)

EFFECT	.5	2	5	SCORE
INJECTION	-	-	-	-
HEMORRHAGE	-	-	-	-
COAGULATION	-	-	-	-

SA.: 0

HET CODE: EGG No.: 18 SUBST. CODE: LT 3
APPL D: 10 APPL VOL: 0.2 ml CONCENTRATION: 1 %
VEHICLE: Olive oil

TIME (MIN)

EFFECT	.5	2	5	SCORE
INJECTION	-	-	-	-
HEMORRHAGE	-	-	-	-
COAGULATION	-	-	-	-

SA.: 0

HET CODE: LT 3 EGG No.: SUBST. CODE:
APPL D: 10 APPL VOL: 0.2 ml CONCENTRATION:
VEHICLE:

TIME (MIN)

EFFECT	.5	2	5	SCORE
INJECTION				
HEMORRHAGE				
COAGULATION				

SA.:

HET CODE: LT 3 EGG No.: SUBST. CODE:
APPL D: 10 APPL VOL: 0.2 ml CONCENTRATION:
VEHICLE:

TIME (MIN)

EFFECT	.5	2	5	SCORE
INJECTION				
HEMORRHAGE				
COAGULATION				

SA.:

HET CODE:	LT 3	SUBST. CODE:	LT 3
APPL D:	10	APPL VOL:	0.2 ml
VEHICLE:	Olive oil	CONCENTRATION:	10 %

MEAN SCORE 6 MEMBRANE REACTIONS (DOSE) : 0.33
MEAN SCORE 6 MEMBRANE REACTIONS (CONTR) : 0.33

ASSESSMENT : "practically non-irritant"

HET CODE:	LT 3	SUBST. CODE:	LT 3
APPL D:	10	APPL VOL:	0.2 ml
VEHICLE:	Olive oil	CONCENTRATION:	1 %

MEAN SCORE 6 MEMBRANE REACTIONS (DOSE) : 0.33
MEAN SCORE 6 MEMBRANE REACTIONS (CONTR) : 0.33

ASSESSMENT : "practically non-irritant"

TOXICITY - TESTING OF ULTRAVIOLET FILTERS BY HET AND HET - CAM

N.P. Luepke (*) and U. Hoppe (#)

(*) Inst. of Pharmacology and Toxicology, Univers. of Muenster
(#) Research and Development cosmed, Beiersdorf AG

Resumen

HET (Hen's Egg Test, Test Huevos de Gallina) y HET-CAM (HET-Chorionallantois-Membrane-Test, Test de la Membrana Corio-Alantoides) son alternativas rapidas y sensitivas de investigaciones en mamiferos y ofrecen informacion con respecto a embrioletalidad, desarollo y crecimiento embrionario, teratogenicidad y toxicidad sistemica incluyendo patologia organica e immunologica y en el caso del HET-CAM ofrece informacion concerniente a substancias quimicas de potente irritacion de membrana. Entre otros las UV-filtros 2,4,6-trianilino-(p-carbo-2-ethylhexyl-1-oxi)-s-triazin (63.5% en 2-ethylhexanoic acid-C16/C18-ester, 'TRI'), octyl-N-dimethyl-PABA ('ODP') y 2-ethoxyethyl-p-methoxy-cinnamate ('EMC') fueron probados bajo los arriba mencionadas condiciones de HET y HET-CAM en una serie de experimentos. Signos de toxicidad dosis- y tiempo-dependientes fueron encontrados en HET y permitieron la classificacion de 'NOELS'. Bajo esas condiciones de test demostro EMC el mas alto grado de letalidad y toxicidad. Por otro lado TRI resulto tener las mas baja respuesta toxica y sin hallazgos teratogenicas comparadas a controles negativos (aceite de oliva) y positivos (vitamina A-acida-trans); esto es cierto tanto para el TRI comercial, como para cada uno de sos ingredientes. En HET-CAM las arriba mencionadas substancias, disoeltas o suspendidas en a- ceite de oliva, no demostraron efectos relevantes de irritacion de membrana. Pruebas de demudacion de la parte ventral del antebrazo de 8 personas voluntarias revelo que 30 minutos posterior a la applicacion de una solucion 0.5% de ODP solo 75% estuvieron sin el estrado corneo, mientras que en el caso del TRI 89% fueron recuperados.

Summary

HET (Hen's egg Test) and HET-CAM (HET-Chorionallantois-Membrane-Test) are rapid and sensitive alternatives to investigations in mammalians and give informations concerning embryolethality, embryonic and growth development, teratogenicity and systemic toxicity incl. organ- and immunpathology and in case of HET-CAM concerning membrane irritation potencies of chemical substances. Among others the UV-filters 2,4,6-trianilino-(p-carbo-2-ethylhexyl-1-oxi)-s-triazin (63.5% in 2-ethylhexanoic acid-C16/C18ester, 'TRI'), octyl-N-dimethyl-PABA ('ODP') and 2-ethoxyethyl-p-methoxy-cinnamate ('EMC') were tested under the a.m. HET- and HET-CAM- conditions in a series of experiments. Dose- and timedependent signs of toxicity were found in HET and allowed the assessment of 'NOELS'. Under the test conditions EMC showed the highest lethality rates and toxicity. On the other hand testing of TRI resulted in the lowest toxic responses at high dose levels and without teratogenic findings compared to negative (olive oil) and positive (transvitamineA-acid) controls; this is true to commercial TRI as well as for the active ingredient alone. In HET-CAM no relevant membrane irritation potencies of the a.m. substances dissolved or suspended in olive oil were observed. Stripping tests at the volar forearms of 8 human volunteers revealed that 30 minutes after application of 0.5% solutions of ODP only 75% were within the horny layer, while in case of TRI 89% could be regained.

postal address:

(*) Institute of Pharmacology and Toxicology, University of Muenster
Domagkstr. 12, D-4400 Muenster, Fed. Rep. Germany

1. Introduction

The increasing variety and number of chemical substances introduced onto the market and also in environment each year and the resulting requirements of protection of human health and human environment have necessitated the monitoring environmental materials (**RTM**) and specimen banking (**ESB**) (1,2,3,4) as well as the development of rapid and reliable methods for toxicity evaluation and risk assessment; this is true also for cosmetics and their ingredients (5). Just so ethical and legal obligations, e.g. animal protection laws, must be taken into account. Chicken embryo models are well known and recommended as screening investigations in testing embryonic development and embryotoxicity; they show a lot of advantages, but nevertheless disadvantages also must be discussed. These basic evaluations were extended and standardized in our laboratories in the last years by the development of **HET (Hen's Egg Test, Hühner Ei Test)** (6,7,8,9). **Hen's Egg Tests** give informations regarding embryolethality, embryonic and growth development, teratogenicity and systemic toxicity, including organ- and immunpathological aspects, and metabolic pathways (10,11). This **HET**-Toxicity-Test is now extended and standardized in the development of **HET-Chorionallantois-Membrane-Test (HET-CAM)** as an alternative to the Draize in vivo rabbit eye test as a membrane irritation test (12,13,14). Regarding the actual discussion, also in the special field of cosmetics, of **RRR** (Refinement, Reduction, Replacement) of animal experimentation it is very important that testing incubated hen's eggs is a borderline case between **in-vivo** and **in-vitro** systems and does not conflict with ethical and legal aspects esp. animal protection laws.

2. Materials and Methods

2.1 Test Substances

The following UV-filters (figure 1) were tested

a) 2,4,6-trianilino-(p-carbo-2-ethylhexyl-1-oxi)-s-triazin (63,5 % in 2-ethylhexanoic acid-C16/C18-ester); "TRI"

b) 2,4,6-trianilino-(p-carbo-2-ethylhexyl-1-oxi)-s-triazin
"TRI" (a.i.)

c) octyl-N-dimethyl-p-amino-benzoic acid
octyl-N-dimethyl-PABA; "ODP"

d) 2-ethoxyethyl-p-methoxy-cinnamate
"EMC"

The materials were of cosmetic quality and were dissolved or suspended in olive oil (pharmacopoe quality) for application. In **HET** the doses ranged from 25 ug to 25 mg/egg. In **HET-CAM** concentrations of 1 and 10 % (in olive oil) were tested.
In the penetration study 0.5 % solutions of TRI and ODP in Ethanol/n-Hexan (75:25) were applied.

2.2 HET

The fertility and hatchability of eggs and the livability of chicken are dependent on a complex interrelationship of ecological factors, among which are the genetic background and the age of the flock, and in part seasonal variations. The White Leghorn has been widely used in experimental studies in the past. The data accumulated during the last years in our laboratory show that the hatchability of White Leghorn (Lohman Selected Leghorn,LSL) eggs is consistent, reproducible and very high and that this breed of chicken is free of known hereditary defects. Eggs to be injected are first candled in order to discard those that are defective; after the elimination of such defective eggs and only use of fresh, fertile eggs, the hatch of control eggs in **HET** is 85 - 95 %. A further restriction is based on the weight of the eggs: all those weighing less than 50 g or more than 60 g are rejected. The eggs were randomized and distributed on groups of 20 or more eggs. The test compounds were dissolved or suspended in the vehicle (olive oil) and administered by a single injection in a volume of 0.1 ml; controls were treated with the same volume of olive oil only. The test compounds were administered by injection in the egg albumen after wipening of the injection area with a sterile gauze pad moistened with a 70 % alcohol solution and drilling a hole in the shell and a further wipening. As soon as the egg has been injected, the hole in the shell is covered with

a surfacer (figure 2). For each injection eggs were treated at two stages of incubation: preincubation (0 hr) and at the fifth day (96 hr); hence there were two test conditions for each compound. The injected eggs are put in the incubator trays with the large end up; the trays are placed in the incubator, which automatically rotates and is maintained at an optimun temperature of 37.5° C (± 0.5° C) and a relative humidity of 62.5 % (± 7.5 %). The eggs are candled on the fifth day of incubation and every day thereafter; nonviable embryos were removed and surviving embryos were allowed to hatch. On the seventeenth day of incubation the fertile eggs were transferred to the hatcher and kept at a temperature of 38.5° C (± 0.5° C) and at a relative humidity of 77.5 % (± 7.5 %) until they hatch. All nonviable embryos and hatched chicken were examined grossly for abnormalities of structural nature as well as for signs of toxicosis (e.g. edema and hemorrhage). Embryos with apparent skeletal defects that could not be readily classified were cleaned and stained with Alizarin Red (modif. Dawson method) to determine the specific anomaly present. For each test condition, the parameters of table I were estimated.

2.3 HET-CAM

Fresh fertile White Leghorn Eggs (Lohman Selected Leghorn, LSL) are used in **HET-CAM-Test**; the eggs are hold in optimized incubation conditions as described in 2.1 (**HET**). In case of **HET-CAM**-Testing on the 10th day of incubation the egg shell is scratched around the air cell by a rotating dentist-sawblade and prepared off. After careful removement of the inner egg membrane the vital, vascular **CAM**, a membrane without sense of pain, is layed open (figure 3); this is a modified technique to that described by Kemper 1958 (12). The test substance, dissolved or suspended, is dropped on the **CAM** in a volume of 0.2 to 0.3 ml; in case of solid test materials 0.1 g is applied and irrigated after 20 sec with 5 ml of warm water. After application the **CAM**, the blood vessels incl. capillary system and the albumen are examined and scored for irritant effects (vascular injection, hemorrhages, coagulation) at 0.5, 2 and 5 min after treatment in a special observation apparatus (figure 3). The numerical time dependent

scores were summed to give a single numerical mean value indicating the irritation potency of the test substance on a scale with a maximum value of 21 (mean of in minimum 4 tests); a rapid risk assessment is possible by a classification scheme analogous to the raw Draize categories (table II).

2.4 Penetration study

TRI (a.i.) and ODP were dissolved in a concentration of 0.5 % in Ethanol/n-Hexan (75:25). 20 ul of this solution (= 100 ug a.i.) were applied on the volar forearms of 8 human volunteers (diameter 1 - 2 cm). 30 Minutes after application the skin was stripped (20x) with Tesa 206 PV. The films were extracted with 2-propanol and the extracts analyzed by HPLC, as follows:

apparatus:	HP 1084 B
column:	RP 8, 12.5 cm, 5 um LiChroSpher;
	guard column RP 8 (10 um) 2 cm
flow medium:	ethanol/water 87:13
	flow 1 ml/min
detector:	var. UV-detector, 310 nm
inj. volume:	20 ul

3. Results

3.1 Embryolethality

All tested pure compounds induced a dose related mortality rate in chicken embryos after application on d 1 and d 5, which allowed the estimation (method of Lichfield and Wilcoxon) of a LD_{50} (table III). In case of commercial TRI (68.5 % a.i.) the mortality rates were very low and a LD_{50} could not be estimated; a mathematical extrapolation showed an LD_{50} of 45 (d 1) resp. 25 (d 5) mg/egg. The results observed must be seen in comparison to a "yardstick" of chemical substances with known toxic potency (table III). In so far TRI, commercial and active ingredient alone, showed the lowest toxic responses, on the other hand EMC resulted in remarkable lethality (the lowest dose of 25 ug/egg led to a lethality of 39 (d 1) resp. 38 (d 5) % of treated eggs).

3.2 Retardation

In all experiments developmental retardation (bone length, hatching weights, s. table II) was found in all higher dosed groups (up to 25 mg/egg), generally combined with increased mortality rates. In our studies, in agreement with other investigations in the past, the test materials are more toxic when given at 96 h than when given at 0 h, irrespective of whether or not a teratogenic response was observed. However, if abnormal development was produced by the chemical, it was enhanced by injection at 96 h, because this time is a period of rapid organogenesis in the developing embryo. The results observed must be seen in comparison with "norm" values by **HET** in LSL eggs (table IV); there are only small differences within different strains of White Leghorn eggs (table V).

3.3 Teratogenicity

There was no evidence of a teratogenic potential of the investigated compounds under the test conditions neither in died nor in hatched chicken. On the other hand the positive control, trans-vitamin A-acid, resulted in the expected rate of malformations.

3.4 Systemic effects

Blood chemistry parameters and organ weights were analyzed in hatched chicken of all groups. In comparison to control-groups and "norm"-values (table IV,V) there were no substance related or dose dependent findings of toxicological relevance.

3.5 Membrane Irritation (HET-CAM)

The a.m. UV-filters (TRI, TRIa.i., ODP, EMC) were tested under **HET-CAM**-conditions in concentrations of 1 and 10 %, dissolved or suspended in olive oil, concerning membrane irritation potencies. Inall cases there were no signs of irritation effects (vascular injection, haemorrhages, coagulation) (figure 4); these investigations resulted in the **HET-CAM**-assessments "practically no irritant".

3.6. No Effect Levels

Up to concentrations of 10% (in olive oil) the a.m. UV-filters showed no irritation effects in **HET-CAM.**
In **HET** the following "NOELS" can be estimated :

	d1	mg/egg	d5
TRI	2.5		1.0
ODP	1.0		0.25
EMC	0.025		0.025.

3.7. Penetration Study

20 ul of a solution (= 100 ug a.i.) of TRI(a.i.) resp. ODP (0.5% in Ethanol/n-Hexan 75 : 25) were applied on the volar forearm of 8 human volunteers (diameter 1-2 cm). 30 minutes after application the skin was stripped (20 x) with Tesa 206PV1. After extractionwith 2-propanol the contents of TRI and ODP were analyzed by HPLC. The investigation revealed, that only 75% of ODP were within the horny layer, while in case of TRI 89 % could still be regained (figure 5). TRI showed a lower resp. slower penetration rate in the stratum corneum than ODP; probable these differences may be founded in the higher molecular weight (~~725~~) and the bulky structure (figure 1) of TRI.

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4. Conclusions

HET (Hühner-Ei-Test, Hen's-Egg-Test) and **HET-CAM (HET-Chorion-allantois- Membrane-Test)** are rapid and sensitive "alternatives" to investigations in mammalians and give informations concerning embryolethality, embronic and growth development, teratogenicity and systemic toxicity incl. organ- and immunpathology and in case of **HET-CAM** concerning membrane irritation potencies of chemical substances .

The UV-Filters **TRI, TRI (a.i.), ODP** and **EMC** were tested under **HET** and **HET-CAM**-conditions.

In HET substance related and dose and time dependent signs of toxicity was found and allowed the assessments of "NOELS". EMC showed the highest lethality rates and toxicity, on the other hand TRI resulted in the lowest toxic response at high dose levels and without teratogenic findings; this is true to commercial TRI as well as for the active ingredient dose.

In HET-CAM no relevant membrane irritation potencies of the tested UV-filters were observed.

Stripping tests at the volar forearms of 8 human volunteers revealed that 30 minutes after application of 0.5 % solutions of ODP only 75 % were within the horny layer, while in case of TRI 89 % could still be regained.

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TABLE I

TOXICITY - TESTING BY H E T

1. Lethality
   LD_{50}

2. Retardation
   a) hatching weight
   b) bone lengths (numerus, ulna, femur, tibia, metatarsus, skull width)
   c) a-b correlation
   d) organ weights (heart, liver, thyroid, thymus, bursa of Fabricius)

3. Teratogenicity
   a) macroscopic-anatomical
   b) clearing and staining of skeleton

4. Systemic Effects
   a) blood chemical parameters (Na, K, Ca, Cl, inorg. PO_4, proteins, creatinine, uric acid, glucose, cholesterol, triglycerides, GOT, GPT, AP, LDH, g-GT, bilirubine)
   b) haematological parameters (haemoglobine, haematocrit, blood cells, coagulation)
   c) organ weights and histopathology

5. Immunpathology
   a) thymus
   b) bursa of Fabricius

6. 1-5 investigations after application on day 1 (before incubation) and on day 5 (96h after beginning)

TABLE II

HET-CAM

Score for Scaling

effect	time (min) 0.5	2	5
injection	5	3	1
haemorrhage	7	5	3
coagulation	9	7	5

Classification Scheme

cumulative score	assessment
- 0.9	pract. no irritant
1 - 4.9	slight irritant
5 - 8.9	moderate irritant
9 -	strong irritant

Table III
Range of Acute Embryotoxicity (LD_{50}) by HET

Substance	d1		d5	
	ug/egg	ppm *	ug/egg	ppm *
Na - Pyrithione	1.5	0.03	0.1	0.002
Zn - Pyrithione	13.5	0.3	0.8	0.02
EMC	110	2	85	1.5
Oxypyrion	2,000	40	750	15
ODP	2,950	60	1,850	35
Piroctone	4,900	100	930	20
2,4 - DAA	7,800	160	2,800	60
TRI (a.i.)	10,600	210	5,350	110
TRI	>25,000	>500	>10,000	>200
DEHP	>100,000	>2,000	>60,000	>1,200

* rounded